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A CONTRIBUTION
TO THE
STUDY OF THE NATURE OF ENZYMES

BY

MAXIMILIAN HERZOG, M.D.

CHICAGO, ILLINOIS

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Preface

When the late Dr. Maximilian Herzog, author of this work, fully realized that his death was imminent, he called me, as President of the Board of Directors of the Municipal Tuberculosis Sanitarium, to his bedside and made known to me his wishes regarding certain manuscripts which dealt with ferments and their nature, the substance of which will be found in the succeeding pages of this publication. This work, Dr. Herzog explained, was the result of twenty-five years of research and study. It was not complete and he knew he would not live to finish it. I immediately set to work to help him prepare his manuscript for publication, and the work of arranging and typewriting the manuscripts was carried on as fast as possible until Dr. Herzog's condition became so serious that further work had to be abandoned.

After Dr. Herzog's death, I appointed a committee to decide on what disposition should be made of the work. The Committee appointed consisted of myself and the following other members:

Colonel Henry I. Raymond, MC., U. S. A., Chairman;
Dr. D. J. Davis, Pathologist, University of Illinois College of Medicine;
Dr. George P. Dreyer, Department of Physiology, University of Illinois College of Medicine;
Dr. A. I. Kendall, Dean, Northwestern University Medical School;
Mr. H. C. Sweany, Senior Chemist, Municipal Tuberculosis Sanitarium;
Dr. Grace S. Wightman, Statistician-Director Publicity and Education, Municipal Tuberculosis Sanitarium.

The problem considered was this: Should we put the work before the public with the understanding that it is incomplete and run the risk of criticism, or should we abandon the work and thereby lose the valuable life work of a careful, conscientious, and tireless investigator, including also many hundred valuable experiments, some of which cost considerable money as well as time? We not only chose the former, but felt in duty bound to do it for the sake of scientific research.

Anyone can realize the difficulty we encountered in attempting to perform the task before us. We were confronted with the problem of taking up an incomplete work left by one man, and placing it before the world in a way that would do the most possible good and yet avoid unmerited criticism of the author. Because of the fact that this problem has been only partially worked out, the following work will indeed be open to a certain amount of inquisitive criticism.

After going into the problem thoroughly, the Committee decided to have the manuscripts reviewed, condensed where necessary, and published as a monograph, and distributed among libraries, laboratories, and educational institutions throughout the country.

The great problem that every one, scientist or layman, has been trying to solve, is the problem of life. How did life originate? To attempt to bridge the gap between inert and living matter is a big undertaking. We can lay out only a few plans or theories, and endeavor to erect the structure, piece by piece.

To prove that life began in the smallest albuminoid particles as soon as they were capable of growth and reproduction would be a complement to either the recapitulation or evolution theories. Certainly the great problem is worthy of consideration.

To some this scheme may seem fantastic, but all great structures must first be planned by an architect. To go about building blindly results in confusion, and so it is necessary to think out many of these problems in theory and then build, or attempt to build, the structure.

Suppose, for example, that we consider the sub-atomic phenomena. A few years ago an atom was thought to be the smallest particle of matter. Now we know that the atom is made up of thousands of positive and negative charges called electrons. A few decades ago it would have been madness to have suggested such a thing as an electron. Yet one does not have to go that far to assume that somewhere between the limit of vision of our microscopes and ultra-microscopes and the largest protein molecule (representing diameters from 0.2 u to .001 u) there exists a world of life as yet unknown to us. Just because our microscopes do not reveal any forms like bacteria, for instance, is not proof that they do not exist. Many diseases are caused by ultra-microscopic organisms. Just as the microscope revealed a new world of life, so many other methods reveal still more.

It has been our aim to condense as much as possible without sacrificing any of the essential material. We found scores of experiments in the older manuscripts, prepared in 1894 and 1895, that were either unimportant or were incorporated in his later work, which are therefore not included.

It is the purpose of this work, therefore, to publish only the results of work bearing upon the problem in question. There are many small groups of experiments, any one of which would make a publication, but their substance has in most cases been incorporated into the work here published.

There is nothing in this monograph that is not original with the author, except possibly certain of the methods of analysis which are mentioned later.

In order to complete the work it became necessary to add a few points in the introduction and discussion of the paper. We might add, however, that they are statements of the author's own ideas and thoughts as they were expressed many times. If there should be some misquotations, please remember that Dr. Herzog is not here to defend them, and there is always a chance of misquoting when the words are not written or taken verbatim.

The work of reviewing and condensing the manuscripts was assigned to Mr. H. C. Sweany, for the reason that he assisted Dr. Herzog in a great part of his work and was in a position to know more about it than anyone else.

John Dill Robertson,
Commissioner of Health.
President, Board of Directors, Municipal Tuberculosis Sanitarium.

A Contribution to the Study of the Nature of Enzymes

ABOUT twenty-five years ago, while studying the blood of a certain acute infectious disease, the cause of which remains unknown even to-day, the author made some observations and from them drew certain conclusions. It is not necessary for our present purpose to discuss here whether these observations themselves were correct or not; they suggested at the time, however, that the disease studied was due to exceedingly small live granules; that these were multiplying in the blood; that the morbid process was to be looked upon as a pathologic fermentation; that every fermentation ultimately was due to such minute, either barely visible or entirely ultramicroscopic, granules; that, in other words, unformed ferments or enzymes were not chemical bodies in the common sense of the word, but something alive, possessing all of the properties of live substances—namely, the power of assimilation, metabolism, growth, and reproduction; that they were, in fact, the real basis of life, the true elementary organisms of all that we call life; and that the latter, in all of its phases, was due to the activities of such exceedingly small live ferment granules.

These, of course, are very bold, revolutionary, and startling ideas. We have become so thoroughly imbued with the belief that trypsin, ptyalin, and other enzymes are purely chemical compounds of complicated, though not yet known formulæ; we are so firmly in the fetters of this fixed idea, that it must be as difficult to the average physiologist or physiological chemist to conceive of the metabolism, growth, and reproduction of an enzyme as to conceive of the metabolism, growth, and reproduction of an ordinary chemical compound like sulphate of copper.

Yet we will be able to furnish in this paper convincing, incontestable, experimental proof of our conception.

At the time when the author first came to the above conclusions (1894), nothing was known of ultramicroscopic, filterable viruses as the cause of such diseases of higher animals as, for instance, foot and mouth disease of cattle, hog-cholera of swine, etc. There had already been described, however, by *Beijerink,*[1] the "mosaic disease" of the tobacco plant, in which the virus could be filtered through clay filters and the disease could be transmitted by the cell free filtrate from plant to plant. The virus, therefore, was called a "contagium vivum fluidum."

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[1] Beijerink: Control of Bact. Abth 11., Vol. V, 1899, p. 77.

After having formed the idea that enzymes were either composed of ultramicroscopic or nearly ultramicroscopic granules, it was thought that it would be very easy to demonstrate this by the use of bacteriologic methods of transfer from one suitable culture medium to another. The enzyme selected for proving the theory was ptyalin of saliva and malt diastase, now often called animal and vegetable amylases. The fermentation product of these enzymes is maltose, which can be very easily demonstrated and determined quantitatively by a number of tests, preferably by using Fehling's solution, volumetrically, or according to Allihn's method, gravimetrically. These amylolytic enzymes, therefore, appeared the most favorable objects for furnishing the experimental proof desired. The first difficulty met with in working with saliva and diastase of malt was bacterial contamination. This, however, was completely overcome in the course of time, because comparative tests demonstrated in carbolic acid and sodium fluoride two strong antiseptics which did not interfere with amylolytic action, even in comparatively strong addition, but even increased it in moderate percentages of carbolic acid and in rather high percentages of sodium fluoride. By the use of these antiseptics, therefore, bacterial contamination could be prevented and the activity of interfering microorganisms could be excluded without loss of amylolytic action. Still, when transplants according to bacteriologic methods were made from fermenting starch solutions to sterile non-fermenting ones, amylolytic action could never thus be transferred in a sufficient and convincing manner. This was then ascribed to the detrimental, inhibiting influence of the fermentation products—dextrines and maltose, present and accumulating.

Experiment Showing the Inhibiting Influence of Maltose in Solutions Undergoing Amylolytic Fermentation

The fermentation products present and accumulating in a starch paste undergoing amylolytic fermentation, as has been known for a long time, act as hindering agents to the further splitting up of the polysaccharids. When this obstacle first confronted the author, accurate experiments to express the inhibiting influence in comparative figures were not available, hence the following experiment was made:

EXPERIMENT NO. A (1894)

Maltose prepared as follows: 50 grams of starch paste were mixed in a flask with about 200 c. c. of cold water. This suspension, well shaken, was poured into 500 c. c. of boiling water. When cooled down to 46° C. (the optimum temperature of amylolytic action of saliva), 100 c. c. of freshly collected saliva was added to the gelatinized starch. The fermentation was then allowed to go on several hours at the above temperature. The dextrines formed were removed; the maltose purified, and used in a comparative series in which definite amounts of maltose were added to flasks containing 1 gram starch gelatinized in water, the whole made up to 40 c. c. To this volume was added 10 c. c. of a 10% fresh saliva—90% sterile water mixture. Fermentation was allowed to go on for 2 hours at 46° C.; all flasks were then placed in the steam sterilizer for a sufficient time to interrupt the fermentation by sterilization of the fluids. The following result was obtained:

Maltose in solution from the start.	*Maltose formed in addition.*
0%	811 milligrams
1%	783 milligrams
2%	453 milligrams
3%	270 milligrams
4%	210 milligrams
5%	66 milligrams
6%	31 milligrams
7%	32 milligrams
8%	trace
9%	trace
10%	trace

This experiment shows the great inhibiting influence of the presence of maltose.

Attempts to propagate the hypothetical ptyalin granules in media containing starch were, therefore, given up after numerous failures or very partial successes only, and starch-free media were substituted. In preparing starch-free nutrient fluids intended for culture media for the hypothetical live ferment granules, free use was made of the observation of *R. H. Chittenden** and his co-workers, who had studied chemical and other factors, favoring, damaging, or completely inhibiting amylolytic action of salivary ptyalin and malt-diastase, and also similar influences with reference to the action of peptic and tryptic enzymes.

The fluid media prepared were distributed to test-tubes or flasks in the usual manner employed in bacteriologic work. They were inoculated, either in very small amounts by platinum loop inoculation, or in larger, measured amounts of saliva, diastase of malt, and extracts from salivary and pancreatic glands. The media prepared generally contained as an antiseptic to prevent bacterial contamination, either carbolic acid (2%)

**Chittenden*: Studies from the laboratory of Physiologic Chemistry, Sheffield Scientific School of Yale College, New Haven, 1885. Subsequently *Effront:* Les Enzymes, Paris, 1899, p. 133ff. His investigator has studied the effect of various substances, such as phosphate of ammonium and of calcium, acetate of ammonium, asparagin, etc., upon amylolytic fermentations.

or sodium fluoride (2½%). The transplants were made in the usual manner.

After a No. 1 test tube or flask had been inoculated it was kept in the incubator or water bath at blood or at higher temperatures. The latter were particularly used in the work with malt diastase (its optimum temperature of amylolytic action is 63° C., that of salivary ptyalin is 46° C.). Transfers to No. 2 test-tubes or flasks were made at intervals varying from a very short time to hours, days, weeks, and months. After a number of transplants had been thus made, up to a No. 10 for instance, and after the lapse of a last time interval, or gradually from time to time, the contents of the test-tubes or flasks were poured over to sterile 1% or 2% starch pastes again containing the antiseptic. The author thus worked for years, employing the same general principle, yet varying the details as to the starch-free culture media again and again. Yet in thousands of experiments he had a moderate success only from time to time.

In a series of 10 transplants, No. 1 added to the starch-paste would produce a good amount of maltose, No. 2 a fair amount, the following transplants, however, only very small amounts or traces, or they were entirely negative. Introducing into the nutrient media prepared, substances detrimental to the proteolytic enzymes, for instance considerable percentages of NaCl, did not much alter the results, though it was at one time strongly believed that the presence of peptic ferments was mainly responsible for the destruction of the amylolytic enzyme. The presence of peptic enzymes was not, however, the main source of the trouble, and it required the efforts of many years before the obstacles to a successful demonstration of the truth were removed.

It was finally recognized that a purely watery solution was quite unfavorable to a multiplication of the ferment granules and it had to be partially replaced by glycerine, which was ultimately used in the culture media in a concentration as high as 35 to 50 volume per cent (with 50 to 65 volume per cent of water). The most favorable percentages of glycerine, as well as the permissible percentages of antiseptics, were ascertained by series of comparative tests, the results being always expressed in amylolytic power; i. e., in yields of maltose. The tables compiled in connection with this work are not given here but are omitted.

Before completely successful transplants could be made it was first necessary to learn that enzyme granules, from the time they are reproduced in the gland until they have formed their last fermentation product, go through a cycle of changes of generation. This term is used here, not in the orthodox zoölogical, but in a restricted sense, as will be more fully explained later. Each fermentation product such as amylodextrin, erythrodextrin, achroodextrin, represents changes of generation occurring in the ptyalin granules, engaged in splitting up the polysaccharide, starch. Each generation of granules possesses the power of assimilation, metabolism, nutrition, and growth; but only one generation possesses the power of reproduction, and that is the generation which is continually forming anew in the gland which furnishes the enzyme. This first generation does not, however, possess the characteristic fermentative power towards a definite substance (starch, proteins, etc.), and this generation of reproducing enzyme granules heretofore has been known as the *zymogene stage* of the enzyme. Let anybody who is familiar with enzymes, who has been engaged in work with them, for the sake of the argument, concede this claim for a moment and he will appreciate at once the enormous difficulties confronting one who wants to prove that enzymes are live multiplying granules or true bioblastic or biophoric units or elementary organisms.

We all have for years been studying the actively fermentative, but non-multiplying, generations; we know well their fermentation products and what is favorable and unfavorable to them. We know very little, practically nothing, of the zymogen which represents the only reproducing elementary organism; we are entirely ignorant of its metabolic products, of influences favorable or unfavorable to it. This is true not only of the zymogen of ptyalin, but also of any zymogen.

The nutrient media used for a long time contained peptone, because it had been shown by *Chittenden* and his co-workers that this substance is very favorable to amylolytic action. Peptone, however, was abandoned and proteid free solutions were prepared, when it became necessary to make determinations of the nitrogen of the enzymes present in the solutions. The formulæ for the nutrient solutions used are somewhat elaborate and they can perhaps be much simplified. The principle underlying the formulæ is to introduce into the fluids substances known to be favorable to amylolytic action; antiseptics inhibiting bacterial development, but not, or only moderately, detrimental to the amylolytic enzymes; and chemical compounds offering N.H.O.H.C.P. and S. in a form easily available to low organisms in their metabolism and reproduction; and, finally, replace part of the water by 35% to 50% of glycerine. One of the first fluids, permitting

The Cultivation of the Amylolytic enzyme granules

and designated in the protocols as formula J50 is the following:

Ammonium Lactate	10.0 c. c.
Sodium Chloride	20.0 grams
Sodium Fluoride	2.0 grams
Pure Carbolic Acid melted	20.0 c. c.
Magnesium Sulphate	0.25 grams

Calcium Chloride	0.1 grams
Acid Potassium Phosphate	1.0 grams
Potassium and Antimony Tartarate	1.0 grams
Morphium Sulphate	0.5 grams
Water and c. p. glycerine, equal volumes enough to make	1000.0 c. c.

Dissolve the various chemicals separately and mix together. Some of these substances are evidently not entirely compatible and when the dissolved calcium chloride, in particular, and the potassium and antimony tartarate are mixed with the rest, a slight cloudy sediment falls out. This is filtered off after the fluid has become cool. The filtered, clear solution is alkaline to litmus and acid to phenolphthalein. No further additions or corrections are made.

After producing evidence to justify our work on this problem, and before going into the detailed experiments, we shall endeavor to throw some light on the subject by stating here the ideas of others. We shall show how the trend of biological thought leans toward the end we have in view. Many workers have had ideas similar to our hypothesis, but none have yet attempted to prove or offer any proof for their contentions.*

General Biologic Considerations

A short time after the more modern conception of the cell had been formed, following the publications of *Schleiden* and *Schwan,* the conviction was expressed that the cell could not be the ultimate morphologic and physiologic unit of life; but that there must exist simpler and smaller elementary organisms which themselves are the building stones of the cell. This idea permeates the entire history of modern biology. *Hule,* as long ago as 1841 stated that the cell possibly was made up of simpler, vital units which would rank between the cell and the chemical molecule. *Bruecke,* while refusing to adopt Hule's conception, in 1861, stated: "We therefore are compelled to ascribe to the cell, aside from the molecular structure of the organic compounds which it contains, another structure, complicated in a different manner, and this is what we mean when we speak of the organization of the cell." Later on Darwin, Spencer, Naegeli, Eisburg, Haeckel, DeVries, Strassburger, Weismann, Wiesner, O. Hertwig, and others, advanced theories, maintaining that the cell must contain simpler organisms since their existence only could explain the problems of cell life, of evolution, and of inheritance. *C. O. Whitman* in his lectures, "The Inadequacy of the cell theory of development," (Vol. 2, p. 112, Boston, 1894) says: "We have long been aware that the cell could not be taken as the ultimate unit of life and every notable effort to account for heredity has led to the postulation of primary elements in comparison with which the cells appear as complex organisms. Since Ernst Bruecke first contended for the organization of the cell in 1861 and the existence of smallest parts as the basis for this organization, we have seen similar ideas in the physiological units of Herbert Spencer, the gemmules of Darwin. We will find the secret of organization, growth, development, not in cell formation, but in those ultimate elements of living matter, for which idiosòmes seems to me an appropriate name. What these idiosomes are, and how they determine organization, form and differentiation, is the problem of problems on which we must wait for more light. All growth, assimilation, reproduction and regeneration may be supposed to have their seat in these fundamental elements. They make up all living matter, are the bearers of heredity, and the real builders of the organism. Their action and control are not limited to cell boundaries."

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*Since this work has been done H. F. Osbourne has published a work on "Origin and Evolution of Life" (New York, Charles Scribner's Sons, 1917), which in many instances is similar to this hypothesis, although his conclusions are drawn by inductive reasoning rather than from any experimental proof.

Hertwig in his book on the cell (English translation, London, 1895) in giving a resume of his conception of the cell, says: "We are forced to the conclusion that the cell is a highly organized body composed of numerous minute different parts, and that hence it is in itself to a certain extent, a small elementary organism. . . . It is, of course, necessary to consider that the hypothetical idioblasts must possess the power of multiplying by means of division like the higher elementary units. . . . Darwin, Naegeli, DeVries, therefore, logically assume that their gemmulæ, particles of idioplasm and pangenæ are both able to grow and to divide. This assumption enables us to draw another conclusion, about the nature of the idioblasts, viz.: that by their very nature they cannot be identical with the atoms and the molecules of the chemist and physicist, for the former are indivisible and the latter, although divisible, split up into portions, which no longer possess the properties of the whole. . . . On this account the idioblasts are not identical with the plastidules, the existence of which is assumed by Esberg and Haeckel."

Wiesner: (die Elementarstruktur der lebenden Substanz, Jena, 1892) states: "The assumption that protoplasm contains organized separate particles which are capable of division, and that in fact it entirely consists of such living dividing particles, is forced upon us as the result of recent inquiry. By means of division of these particles, growth is brought about and all the vital processes occurring in the organism depend upon them. They must therefore be considered the true elementary organs of life."

Watase: (The Nature of Cell Organization, Biol. Lectures, vol. 2, Boston, 1894), a pupil of C. O. Whitman, states: "The behavior of the cytoplasm thread or net work suggests that it is formed of a group of small living particles, each with the power to assimilate, to grow and multiply by division. The chromosome in the same way is itself a colony of minute organisms of another kind, each endowed with similar attributes of vitality. The media in which they live, the cytoplasm and caryolymph, are the media in which they breathe, from which they derive their nourishment, or within which they deposit the products of their metabolism. The reason why the cell as a whole assimilates, grows and divides is ultimately due to the fact that the minute particles which compose the cytoplasm and the chromosomes are endowed with these functions."

While Darwin and other biologists assumed the existence of elementary units without entering into any morphologic considerations, there came later on a number of investigators who attempted to find such units by microscopic methods and to reduce the structure of protoplasm to component simple morphologic elementary bodies. A number of theories of the morphologic structure of protoplasm and of simple elementary units were propounded especially by *Fleming, Altman,* and *Bütschli.* M. Heidenhain* in criticising these theories, very properly and in strong terms insists upon the futility of such attempts which search for the ultimate organic units of life as something of necessity visible. He points out how irrational it is to suppose, *a priori,* that these ultimate units of life should fall within the limits of the power of the microscope; and he holds that we must reckon with the probability of an ultimate metamicroscopic structure of the cell and of the protoplasm. *Pfitzner* observed in 1882 that chromatin granules of the karyosomes of cells of salamandra divided, and that, in fact, the division of the chromosome depended upon the division of such granula. This observation subsequently was confirmed by Fleming and others. *Brauer* (Arch.f.mikroscop Anatomie, vol. 42, p. 203) in studying spermatogenesis of ascaris meyalocephala, noticed the same division of chromatin granula. He looks upon this division as an independent act of reproduction and says that he does not look upon chromosomes as independent individuals, but only as the components of innumerable, small chromatin granules. The latter only he considers as truly individual units.

In Wilson (The Cell in Development and Inheritance, New York, 1900) we frequently find the idea expressed that the chromatin granules might belong to the much-looked-for elementary organisms. The author, for instance, says (p. 114): "These observations certainly lend strong support to the view that the chromatin is to be regarded as a morphologic aggregate, as a congeries of or colony of self-propagating elementary organisms, capable of assimilation, growth and division."

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*M. Heidenhain: Plasma und Zelle. Theorie der kleinskn Theilkoerper oder Protomerentheorie. Jena 1907, p. 488.

Wilson also considers that possibility that what we can see does not yet show as the true elementary organism, and he states further on (p. 292): "Yet we may still inquire whether the power of division shown by such protoplasmic masses as plastids, chromosomes, centrosomes, nucleoli and nuclei, may not have its root in a like power residing in ultimate protoplasmic units of which they are made up."

Could we accept such a view, we might more easily meet some puzzling cytological difficulties. It may, however, again be pointed out that it would be quite irrational to conclude that the smallest visible granules first come into existence when they first come within view of the microscope. The homogeneous substance must itself consist of granules still smaller. The real question is not whether such ultra-microscopic bodies exist, but whether they are permanent organized bodies, possessing besides the power of growth, also the power of division.

The belief that visible cell granula, demonstrable by various staining methods, are the looked for hypothetical elementary organisms, found its strongest advocate in the skillful and resourceful histologist *Altman.* The fundamental principle advocated by this investigator is the following: The cell is not an elementary morphologic and biologic unit from which can be derived directly the physiologic activities of the organism. The cell is, both from the morphologic and the physiologic view, a complex structure; it is a colony of granules; i. e., of live beings, or bioblasts, which may be compared to free living microorganisms. The cell granules or globules possess independently the property of assimilation, growth, and especially reproduction, and they possess the property of differentiation so that they can take upon themselves various functions of the cell. Even Altman, however, conceded in later publications that the visible granules might take their origin from still smaller invisible granules, although he insists upon looking at the visible granules as elementary organisms. It is rather unfortunate to compare and place side by side, the hypothetical elementary biophoric units and unicellular microorganisms like bacteria, because the bacterial organism, as everybody who has done much bacteriological work knows, is not at all a very simple body and is no more a real elementary organism or bioblast than a tissue cell of a higher multicellular being. A very important objection against Altman's claims, as pointed out by *Verworn* (Allgemeine Physiologie Jena, 1897, p. 67) is the following: Altman has failed to demonstrate that his granula show the general functions of life, and without such a demonstration one cannot speak properly of an organism. Verworn, however, raises against Altman's granular elementary organisms, an objection which is not at all valid; namely, that it is not permissible to speak of an elementary organism in a cell unless we are acquainted with free living elementary organisms of this type. This is not sound reasoning in itself, because there might be an elementary biophoric unit in the cell which does not exist anywhere free in outside nature; or such units may exist, live, and multiply, outside of the cell, but they might heretofore have evaded detection. The latter, of course, is what really has occurred in the past. Verworn, however, could not see it thus, and his final conclusion is that the cell is the individuum of the lowest order, that it is indeed the elementary organism itself.

Wiesner (quoted from Heidenhain) believes in the existence of smallest ultra or "meta-microscopic" bodies, representing the last ultimate limit, where divisibility of the living substance ceases, because mere fractions of the ultimate unit will no more possess the character of protoplasm or of a live body which can multiply by division. O. Hertwig has accepted Wiesner's theory. Heidenhain calls those hypothetical smallest, ultimate, "meta-microscopic" bodies "protomeres" and he believes every form of growth to be due to the division of these bodies. He also holds that the "protomeres" are the cause of the universal automatism of live matter and that they possess the power of metabolism, of assimilation and dissimilation, of increase in mass, and divisibility. They also possess individuality; they keep themselves alive by metabolism; and they must be looked upon as a biochemical system which, on the basis of its structure, is able to undergo cleavage and division.

Wilson, p. 431. "In its physiological aspect, therefore, inheritance is the recurrence in successive generations of like forms of metabolism and this is effected through the transmission from generation to generation of a specific substance or idioplasm which we have seen reason to identify with chromatin. The validity of this conception is not affected by the form in which we conceive the morphological nature of the idioplasm—whether simply a mixture of chemical substances, as a microsome of invisible germs or "pangens," as assumed by DeVries, Weisman and Hertwig; as a storehouse of specific ferments as Driesch suggests; or as a complex molecular substance grouped in "miscellæ," as in Naegeli's theory."

The contribution of *Beijerink* entitled: "Uber ein Contagium vivum fluidum als Ursache der Fleckenrankheit der Tabaksblaetter" (Centralblatt fuer Bacteriologie, Abth. 11 Vol. V, 1899, p. 77) is not very often quoted, yet the vegetable disease he described represents the first morbid condition in which an invisible, filterable, ultramicroscopic virus was recognized and fully established as the etiological factor. Beijerink was not the first to study this vegetable disease—this was first done by *Adolph Mayer* in 1887, who established its contagious nature. In the same year Beijerink tried to demonstrate ærobic or anærobic bacteria as the cause of the disease, but failed. Later he established the filterable nature of the virus; the explanation, however, in which he tries to give his observations is not at all correct.

Hertwig, p. 339: "Just as the plant or animal can be divided into milliards of elementary parts; viz.: cells, so each cell is composed of numerous small hypothetical elementary particles."

We must also look upon the intercellular substances of multicellular organisms as true live substances, in other words, as composed of elementary biophoric granules. *Virchow,* when developing his cellular pathology and his dictum "omnis cellula e cellula," found something in the nature of a stumbling block in the intercellular substances of the body, and he then formed the conception that intercellular substance, as a mere product of cell activity, possesses no true life itself, and was entirely depending in its nutritional activities upon the cells originally furnishing such substances by excretion, deposit, etc.

M. Heidenhain protests against a conception of life of one structure which is only borrowed of some other structure, and he very properly remarks that according to the true conception of life, live matter must be something always active, preserving its own structure by its own efforts or functions, and not by the functions of another structure. Hence *Heidenhain* speaks of the automatism of life. Some of the intercellular substance of the body is, as we all know, composed of ordinary chemical matter, and this of course is not live substance. But wherever we find an intercellular substance, capable of true nutritional changes, we must be dealing with live, assimilating and reproducing ferment granules. This, of course, opens up an entirely new field and points out the fact that a large part of the body of multicellular beings is composed of live substance not present in the form of cells, but in the shape of elementary units existing in the body independently of the cell organization.

General Remarks About the Following Experiments

The original starting materials used in the following experiments were saliva, extracts or powders from salivary glands, hogs' and dogs' pancreas, etc. In spite of the fact that both the nutrient fluids and the starch gelatines practically always contained antiseptics, all antiseptic precautions were observed in the preparation of the gland extracts or powders, the nutrient media, the starch pastes, etc. The latter not only contained antiseptics, but were, also, generally sterilized by continuous or fractional sterilization in the steam sterilizer. The nutrient fluids were similarly treated. The saliva used was collected fresh from the mouths of several persons; it was mixed in sterile bottles; was shaken, without the addition of sand or anything else, in a shaking machine for 30 to 60 minutes; and was then filtered rapidly through filter paper, with the aid of a water suction pump. The filtering saliva either flowed directly into a measured volume of pure sterile glycerine, containing from 1 to 2 per cent of carbolic acid, or it was subsequently mixed with this fluid. In each case the mixture was made so that equal parts of the filtered saliva and carbol glycerine were present. Such a mixture can be kept for many months in the refrigerator and it will lose its amylolytic power very slowly. The saliva thus prepared was used at shorter or longer intervals after its collection. In some experiments unmixed, fresh, filtered saliva was used. The salivary and pancreatic glands were either ground up in a meat grinder or in a mortar with quartz sand and silicon powder and expressed by a hydraulic press, or were shaken after trituration and after mixture with a preserving (carbol-glycerine) or nutrient fluid (formula J50 or others) which was then filtered through filter paper by the aid of a suction pump. For other experiments an acetone-dry powder obtained by the method of *Rapp and Buchner* in the preparation of acetone-dry yeast powder was employed. The starch pastes generally contained 1 gram of starch gelatinized in 40 c. c. of water containing 2% carbolic acid or 2½% fluoride of sodium.

Such uniformly gelatinized starch pastes are prepared as follows: One dram dry, acid free starch is weighed off and placed into a 100 c. c. Erlenmeyer flask. 40 c. c. of a cold water 2% carbolic acid solution or 2½% solution of NaFl are added. The contents of the flask are then carefully gelatinized over the small open flame of a Bunsen burner by gradual heating to boiling and under continual shaking. The flasks are then placed in the steam sterilizer. One half hour is considered sufficient, but they may be left in longer.

The nutrient solutions containing saliva or gland extracts were usually added in amounts of 10 c. c., and since these fluids also contained the antiseptics in the same percentages, the amylolytic fermentations occurred in the presence of 2% carbolic acid or 2½% NaFl; hence there was no danger of bacterial or similar contamination to which might be ascribed sugar formation from the starch. The use of suction pipettes is to be avoided on account of its danger of contamination with saliva from the mouth, and exact amounts must either be measured out by pipettes provided with rubber bulbs, or by small cylindrical graduates.

Fermenting starch solutions kept in the water bath or incubator were frequently shaken to expedite the saccharification. The determinations of the amounts of maltose formed were made by the volumetric method and by the use of Fehling's standard solution. The yield in sugar was always calculated as maltose (154 milligrams of maltose reduce all of the copper hydrate of 20 c. c. of Fehling's solution). The nitrogen determinations were made by methods to be described below. The absence of bacteria or other microorganisms which might have an amylolytic power was demonstrated by the inoculation of various culture media under aerobic and anaerobic conditions.

EXPERIMENT NO. XIX (SEPTEMBER 30, 1917)

Ten c. c. of a saliva mixture—collected August 18, 1917, and kept in the refrigerator, containing equal parts of saliva and 1% Phenol-Glycerine, were poured into a sterile, perfectly dry, 250 c. c. volumetric flask, and this was made up to the mark with nutrient fluid formula J50.

Ten c. c. of the same saliva mixture were placed into another 250 c. c. volumetric flask and the contents made up to 250 c. c. with 2% water-Phenol Solution.

Ten c. c. out of each of the two flasks were then poured into 100 c. c. Erlenmeyer flasks containing 40 c. c. of a 2% Phenol-Water, 1 gram starch paste. The two flasks were next placed into a continuous self-regulating stationary water bath at 40° C. for 1 hour and 20 minutes. The flasks, after removal from the water bath, were rapidly brought to boiling over the open flame of a Bunsen burner, their contents then filtered through filter paper into 100 c. c. volumetric flasks, the filters repeatedly washed with hot distilled water, the contents after cooling made up with distilled water to exactly 100 c. c. The two tests made right at the beginning of the experiment furnished the figures expressing the initial amylolytic power in one test in the presence of 2% carbolic acid and glycerine only; in the second test in the presence of a number of substances favoring amylolytic action. After the initial tests had been started the 250 c. c. flask with the saliva-nutrient fluid mixture was placed in the incubator and kept there at the prevailing temperature of 37°-38° C. After definite intervals of time, 10 c. c. were always removed from the 250 c. c. flask and poured into 100 c. c. flasks containing in 40 c. c. of 2% Phenol-water the usual amount of 1 gram of starch. These flasks were always kept in the water bath at 40° to 41° C. for a definite period of time, and they were then treated as described above for the initial tests.

The following table gives the maltose formed and thus furnishes comparative figures as an expression of the amylolytic power represented by 10 c. c. at various time intervals.

	Result of Maltose Determinations	*Maltose formed* milligr.
1*a*	2% Phenol Solution Mixture imm. after prep. ferm. 1 hr. 20 min.	126.2
1*b*	Nutrient fluid J50 Mixture imm. after prep. ferm. 1 hr. 20 min.	141.6
2	Nutrient fluid J50 Mixture incubation 5 hrs. 20 min. ferm. 1 hr.	261.9
3	Nutrient fluid J50 Mixture incubation 21 hrs. 35 min. ferm. 1 hr.	299.6
4	Nutrient fluid J50 Mixture incubation 24 hrs. 50 min. ferm. 1 hr.	311.7
5	Nutrient fluid J50 Mixture incubation 36 hrs. 5 min. ferm. 1 hr.	322.2
6*a*	Nutrient fluid J50 Mixture incubation 51 hrs. 35 min. ferm. 1 hr.	334.8

6*b* Nutrient fluid J50 Mixture incubation 51 hrs. 35 min. ferm. 1 hr. 20 min.351.6
7 Nutrient fluid J50 Mixture incubation 75 hrs. 0 min. ferm. 1 hr. 20 min. 319.3
8 Nutrient fluid J50 Mixture incubation 81 hrs. 45 min. ferm. 1 hr. 20 min. 288.4

The result of the above mentioned experiment is the following: Ten c. c. dilute mixed saliva were incubated with 240 c. c. nutrient fluid formula J50; from time to time during incubation the amylolytic action of 10 c. c. of the mixture was tested and it was shown that the amylolytic power during 51 hours had increased from an original of 141.6 milligrams to 351.6 milligrams of maltose formed.

The above mentioned experiments are typical of what occurs generally when saliva is incubated with nutrient fluid J50; namely, a rise in amylolytic power which may be very marked or which may be rather small. The increase generally after a few days reaches a maximum, then the values go down. The results, however, are not always so regular as in the above experiments—there may be a rather irregular curve with more than one apex.

The result of the above experiment, showing increase of amylolytic power over a period of several days, admits of two interpretations:

(1) Nutrient formula J50 represents a mixture in which the amylolytic ferment can be well kept for a number of days and in this mixture the zymogen of ptyalin develops rather gradually but with fair rapidity into the active enzyme; hence what the author has been doing is to develop in his so-called nutrient fluid a heretofore more or less unknown zymogen of human saliva into the active enzyme.

(2) The second interpretation, which we will endeavor to prove, is that ptyalin is not an unorganized chemical compound; but is a live ultramicroscopic ferment granule, an elementary organism; and it assimilates, grows, and multiplies, or propagates, not only under natural conditions in the salivary glands, but also in the author's artificial culture medium, formula J50.

Experiment No. XX

Experiment No. XX is really an integral part of the preceding one, and was started simultaneously with it. When the 250 c. c. flask containing the saliva nutrient fluid mixture was incubated, a 100 c. c. volumetric flask was prepared in the following manner. The flask was half filled with nutrient fluid J50 and the funnel was left in the flask. Its contents were then heated to boiling and 4 c. c. of the saliva mixture were then allowed to run into the boiling fluid; the funnel was washed out with more hot nutrient fluid J50 and the contents of the flask, when cool, were made up to 100 c. c.

This flask was kept as a control in the incubator with the 250 c. c. flask. A third flask prepared contained 1 gram of starch gelatinized in 25 c. c. of 2% Phenol-water. On the fifth day of incubation, and after the contents of the saliva-nutrient fluid in the 250 c. c. flask had shown the strong increase in amylolytic power, 25 c. c. of its contents were poured over to the flask containing the 25 c. c. of starch paste, and saccharification was allowed to go on several hours, until quite complete; the contents of the flask were then brought to boiling, filtered, and made up to exactly 50 c. c.

Determination of proteid nitrogen* was then made of the contents of the three flasks.

Flask A contained the saliva which had at once been sterilized by boiling.

Flask B contained the saliva, mixed with nutrient fluid J50 which had been left unboiled and had been incubated at 37° C. for a number of days.

Flask C contained 25 c. c. of a 1 gram starch paste which had been subjected to the amylolytic action of 25 c. c. from the mixture in Flask B.

The Method of Determination of Nitrogen Employed in the Above and Other Experiments Reported in this Paper is the Following

An aliquot part of the original was taken, and the coagulable proteids thrown down by 10 volumes of methyl alcohol c. p. containing 1/20 saturated $Zn.Cl._2$ c. p. This alcohol-Zn.Cl. mixture was added slowly and stirred; after it was made up to 50 c. c. it was allowed to stand 20 minutes for complete precipitation; then it was centrifugalized at high speed for 5 minutes. The precipitate was easily separated from the liquid, because it remained in the bottom of the tube in a gummy mass; the liquid was poured off and the tube was drained completely. The contents were then dissolved in 1 c. c. of concentrated H_2SO_4 c. p., transferred to a Jena glass test tube, and digested, aerated and determined according to a slight modification of Folin's method. This modification is the same as the original, except that the nitrogen is determined in smaller amounts; instead of using a test-tube 24x200 mm. for collecting the nitrogen, a test-tube 16x150 mm. is used; 5 c. c. of $\frac{n}{20}$ H_2SO_4 are placed in this test-tube, corks and absorption bulbs fitted in the same as in the original method; the nitrogen is carried over in the current of air; care must be taken that bubbles of air do not come faster than 1 or 2 per second. After 1 hour of this aeration the contents of the tube are Nesslerized by using 1 c. c. of Nessler-Winkler solution, diluting it to 4 c. c., mixing it with unknown solution, making the total volume to 10 c. c., and reading the results in a Duboscq colorimeter against a known standard.

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*These and other determinations of proteid nitrogen were made for me by my assistant, Mr. H. C. Sweany, Senior Chemist of the Municipal Tuberculosis Sanitarium. I am pleased herewith to express to him my thanks for the conscientious work he has performed under my direction in connection with this research.

Result of Determinations of Proteid Nitrogen

Proteid Nitrogen in 5 c. c. fluid

Contents of Flask A, Saliva boiled (control flask), None demonstrable in appreciable amount.

Contents of Flask B, Saliva (unboiled) Nutrient fluid mixture, 0.26 mgrs. per 5 c. c.

Contents of Flask C, Starch converted by amylolytic action, *(see remark).

Experiment No. XXI

Another test similar to the one described above was made, but amylolytic control tests were not made. Two 100 c. c. volumetric flasks received each 0.1 c. c. of nutrient fluid formula J50. The contents of one flask were brought to boiling at once. Both were kept in the incubator for 5 days, then determination of proteid nitrogen was made with the following results.

Boiled Control proteid N. per 1 c. c.	None demonstrable
Non-boiled test proteid N. per 1 c. c.	0.1 milligram

Experiment No. XXII (December 13, 1917)

For this experiment another nutrient solution (formula M35) was prepared and used. Formula M35 contains the following substances per 1000 c. c.:

Sulphate of Morphia	0.5
Phosphate of Ammonium	5.0
Phosphate of Calcium (tribasic)	0.1
Acetate of Aluminium	2.5
Asparagin	0.5
Sodium Chloride	5.0
Sodium Fluoride	25.0
Water	65 vol.%
Glycerine	35 vol.%

enough to make 1000 c. c.

The various salts, etc., are dissolved separately in the glycerine-water mixture, the solutions are then mixed, made up to 1000 c. c., heated over an open flame, cooled to 15-20° C., then filtered through filter paper. The resultant solution is markedly alkaline to litmus, very faintly alkaline to phenolphthalein.

2.5 c. c. fresh, shaken, and filtered saliva were placed in a 250 c. c. volumetric flask, plus 247.5 c. c. of the above solution. 1.0 c. c. of the same saliva was next allowed to flow into a 100 c. c. volumetric flask containing about 50 c. c. of boiling nutrient solution M35. After cooling, this flask was made up to 100 c. c. with solution M35.

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*The reading of the amount of nitrogen was not very satisfactory, because the final fluid was not quite clear. The reading gave a value of about 0.07 mgrs., hence a value certainly not higher than that obtained for the contents of flask B.

0.1 c. c. of the same fresh saliva, plus 9.9 c. c. of the above solution M35 were then added to a starch paste; viz.:

1.0 grams starch gelatinized in 40.0 c. c. of 2½% watery Fluoride of Sodium solution.

This flask was kept in the water at 41-42° C. for 2 hours. Maltose formed after 2 hours' fermentation, 294.5 mgrs.

The two volumetric flasks:

250 c. c. containing 2.5 fresh saliva and

100 c. c. containing 1.0 fresh saliva (boiled at once).

were then kept in the incubator at 35° to 37° C. for several weeks and their amylolytic power tested from time to time. The 100 c. c. flask never showed any amylolytic power. Test for amylolytic power made as usual, 10 c. c. of 250 c. c. flask added to 1 gram starch, 40 c. c. 2½% watery NaFl solution, kept in water bath at 41° to 42° C. for 2 hours.

Amylolytic Power

Original of 0.1 c. c. fresh saliva plus 9.9 c. c. formula M35 2 hours	294.5 mgrs. maltose
After 1 day's incubation of nutrient fluid M35	269.3 mgrs. maltose
After 2 days' incubation of nutrient fluid M35	229.2 mgrs. maltose
After 3 days' incubation of nutrient fluid M35	244.4 mgrs. maltose
After 4 days' incubation of nutrient fluid M35	263.9 mgrs. maltose
After 5 days' incubation of nutrient fluid M35	251.6 mgrs. maltose
After 13 days' incubation of nutrient fluid M35	271.2 mgrs. maltose
After 17 days' incubation of nutrient fluid M35	160.4 mgrs. maltose
After 27 days' incubation of nutrient fluid M35	125.2 mgrs. maltose
After 36 days' incubation of nutrient fluid M35	273.1 mgrs. maltose

This marked increase in amylolytic power, after the initial decrease, was so surprising that several more amylolytic tests were made the same day, about 6-7 hours after the above last test had been made. The following tests made at 41°-42° C. in the water bath. All show an increasing amylolytic power.

After 36 days and 6 hrs. incubation of nutrient fluid 1 gram starch in 2½% NaFl. sol. 287.3.

After 36 days and 6 hrs. incubation of nutrient fluid 1 gram starch in 2½% NaFl. sol. 284.1.

After 36 days and 6 hrs. incubation of nutrient fluid 1 gram starch in 2% Phenol sol. 280.0.

After 37 days' incubation of nutrient fluid 1 gram starch in 2½% NaFl. sol. 300.2.

After 37 days' incubation of nutrient fluid 1 gram starch in 2% Phenol sol. 326.3.

After 38 days' incubation of nutrient fluid 1 gram starch in 2½% NaFl. sol. 306.8.

After 38 days' incubation of nutrient fluid 1 gram starch in 2% Phenol sol. 333.3.

After 39 days' incubation of nutrient fluid 1 gram. starch in 2% Phenol sol. 339.2.

On January 21, 1918; i. e., 39 days after the experiment had been started, and after the two flasks had been kept this period of time in the incubator at 35° to 37° C., a Nitrogen determination was made of the fluid of the two flasks, which had both received 1% of fresh saliva with the following result:

Flask boiled at once....................Proteid Nitrogen per 1 c. c. = mere trace
Flask which contained the Nutrient fluid which exhibited the increasing amylolytic power.................................Proteid Nitrogen per 1 c. c. = 0.09 mgrs.

The above and the following proteid nitrogen determinations were made by the Gulick method of analyzing for nitrogen in small quantities. The method has been somewhat modified by my assistant, Mr. H. C. Sweany.

To determine nitrogen in the traces required by the experiments outlined it was necessary to devise a method whereby nitrogen could be analyzed to 0.02 of a milligram. Gulick's direct method was finally chosen, because it enabled one to work with smaller quantities than any other.

The Reagents and apparatus required are:

1. Gulick-Nessler-Winkler solution (see Hawk's Phys. Chem. p. 490).
2. Gulick's digestion mixture.
3. Absolute nitrogen free distilled water for use and for rinsing all glassware and pipettes after they have been digested in a sulphuric acid bath.
4. Small flasks designed on the pattern of Gulick's flask, but smaller and having several points of advantage.

To make the test, 5 c. c. of the sample are carefully pipetted into a 50 c. c. graduated centrifuge tube; to this is added slowly 45 c. c. of 10% $ZnCl_2$ dissolved in methyl alcohol. The mixture is stirred with a clean glass rod and set aside for 15-20 minutes, when it is centrifuged 2 minutes at 2000 r. p. m. After this the alcohol-$ZnCl_2$ may be drained off, leaving the albuminous mass in the bottom of the tube. To this is added enough of Gulick's digestion mixture to put it in solution. Sometimes stirring or gentle heating is necessary. If 5, 6, or 7 c. c. of the mixture are used, then the whole is made up to 10 c. c., and ¼ to ½ c. c. of this is placed in the micro-digestion flask mentioned above, and digested. It requires the flame of a micro-burner about 1 cm. high. The tip of the flask must just touch the end of the flame, and the operator must see that the bottom does not get too hot. It is best to lift the flask out when the bottom gets dry; the H_2SO_4 condenses at the top and runs back. Then the flask may be put in the flame again. After 8-10 minutes the flask clears and then another minute will complete the digestion. Before the flask gets cool 5 c. c. of distilled water are run in from a pipette, the mixture allowed to cool and then 2.5-5 c. c. of the Gulick-Nessler-Winkler solution are used (depending on the amount of digestion mixture started with). If ¼ c. c. of the digestion mixture is used, 2.5 c. c. of the G-N-W solution must be used, etc. The way to mix the solutions is to put them in separate tubes and pour them suddenly together. This prevents the formation of a turbidity. The contents of the tube are now made up to 10 c. c. in a graduated flask with distilled water, and read in a colorimeter against a standard of $(NH_4) SO_4$ that has been treated in the same manner as the unknown; i. e., it is digested and Nesslerized in the same way.

When it becomes necessary, the dilution can be made to 5 c. c. or even less, and read by matching against a standard in small test-tubes having the same volume as the unknown. This method, however, is not so accurate.

The Oxidation flask is made by drawing out the end of a Jena test-tube, rounding the point, and constricting the tube near the bottom. This constriction prevents frothing, holds more heat, bends the tube, and possesses several other advantages.

There had been in the above experiment, No. XXII, a very considerable increase in the amount of proteid Nitrogen in the flask containing the unboiled amylolytic ferment. As seen from the above table, the amylolytic power of ptyalin in nutrient fluid M35 first decreased very much and then from a low level rose beyond the original amylolytic power. The reason for this peculiar behavior was the following: Formula M35 is quite alkaline. It was ascertained that the alkalinity of 100 c. c. of this nutrient fluid M35 was equal to 5.5 c. c. of Normal phosphoric acid (H_3PO_4). In the following experiment the behavior of the amylolytic power in this strongly alkaline solution was compared with the amylolytic power as developed in the nutrient fluid M35 after it had been neutralized with H_3PO_4.

EXPERIMENT NO. XXXII (JANUARY 23, 1918)

Saliva freshly collected, shaken and filtered: 2.5 c. c. of the fresh saliva added to 247.5 c. c. of a freshly prepared nutrient fluid, formula M35. This fluid was not quite as alkaline as the one used in the preceding experiment, its alkalinity per 100 c. c. was equal to about 5 c. c. of phosphoric acid. The 250 c. c. flask thus prepared was placed in the incubator, with it a control-flask containing 1 c. c. saliva plus 99 c. c. formula M35; contents had been boiled at once.

Test of original amylolytic power of saliva: 0.1 c. c. of the fresh saliva, plus 9.9 of nutrient fluid formula M35, added to 1 gram 40 c. c. 2½% NaFl starch paste; the same amount added to 1 gram 40 c. c. 2% Phenol starch paste. Flasks kept in water bath at 41° to 42° C. for two hours. Yield in maltose as expression of amylolytic power:

2½% NaFl starch paste	295 mgrs. maltose
2% Phenol starch paste	293.9 mgrs. maltose

Jan. 23, 1918. Part of the freshly prepared nutrient fluid formula M35 was completely neutralized with phosphoric acid. Then 100 c. c.

and 250 c. c. flasks were prepared, exactly as above. Besides a fifth flask was prepared containing nothing but 100 c. c. of nutrient fluid M35; this was also kept in the incubator as a complete blank. The initial test of the amylolytic power of the saliva used, mixed with the neutralized nutrient formula M35 gave the following values:

2½% NaFl starch paste	321.5 mgrs. maltose
2% Phenol starch paste	316.9 mgrs. maltose

From time to time 10 c. c. of the contents of the 250 c. c. flasks kept in the incubator were removed and tested on 1 gram starch made to a paste having a volume of 40 c. c. as described on page 18, both 2½% NaFl and 2% Phenol. Temperature of water bath, 41° to 42° C.; time, 2 hours.

A—Tests of the Alkaline Fluid

	2½% NaFl.	2% Phenol
Jan. 23, original test	295.0 mgrs.	293.9 mgrs.
Jan. 24, after 1 day's incubation	209.8 mgrs.	245.2 mgrs.
Jan. 25, after 2 days' incubation	227.5 mgrs.	280.0 mgrs.
Jan. 26, after 3 days' incubation	237.3 mgrs.	313.6 mgrs.
Jan. 27, after 4 days' incubation	221.3 mgrs.	284.1 mgrs.
Jan. 28, after 5 days' incubation	237.7 mgrs.	308.0 mgrs.
Jan. 29, after 6 days' incubation	227.5 mgrs.	264.3 mgrs.
Jan. 30, after 7 days' incubation	220.0 mgrs.	270.0 mgrs.
Feb. 1, after 9 days' incubation	231.0 mgrs.	286.3 mgrs.
Feb. 4, after 12 days' incubation	213.3 mgrs.	296.7 mgrs.
Feb. 7, after 15 days' incubation	212.4 mgrs.	295.6 mgrs.
Feb. 18, after 26 days' incubation	339.2 mgrs.	388.9 mgrs.

B—Tests of the Neutral Fluid

	2½% NaFl.	2% Phenol
Jan. 23, original test	321.5 mgrs.	316.9 mgrs.
Jan. 24, after 1 day's incubation	267.4 mgrs.	289.5 mgrs.
Jan. 25, after 2 days' incubation	284.7 mgrs.	273.0 mgrs.
Jan. 26, after 3 days' incubation	292.2 mgrs.	364.1 mgrs.
Jan. 27, after 4 days' incubation	275.0 mgrs.	364.9 mgrs.
Jan. 28, after 5 days' incubation	261.5 mgrs.	353.2 mgrs.
Jan. 29, after 6 days' incubation	234.1 mgrs.	300.8 mgrs.
Jan. 30, after 7 days' incubation	296.2 mgrs.	339.2 mgrs.
Feb. 1, after 9 days' incubation	283.1 mgrs.	308.6 mgrs.
Feb. 4, after 12 days' incubation	296.7 mgrs.	352.4 mgrs.
Feb. 7, after 15 days' incubation	295.0 mgrs.	349.2 mgrs.
Feb. 18, after 26 days' incubation	308.0 mgrs.	475.3 mgrs.

Since the contents of the 250 c. c. flasks had been almost exhausted, the tests of the amylolytic power could not be continued beyond February 18; the remaining amounts of fluid had to be reserved for nitrogen tests.

From the above tests the following is shown: A neutral medium (M35 neutralized) is much better than a decidedly alkaline one of the same composition, except as to correction. In fermenting starches, the presence of 2% Phenol is more favorable than the presence of 2½% NaFl. In both series there was first a loss of amylolytic power, followed finally by a rise. The latter was quite considerable in the neutral solution, more moderate in the alkaline solution.

On February 18, after the amylolytic power on that date had been recorded, the material left in the flasks was removed from the incubator, placed in the refrigerator, and the next day 5 determinations for proteid Nitrogen by Gulick's method were made as follows:

	Proteid Nitrogen per 1 c. c. fluid
No. 1 Blank control	none
No. 2 Control M35 alkaline boiled at once	0.0257 milligrams
No. 3 Control M35 neutral boiled at once	0.0259 milligrams
No. 4 Flask containing live ferment in alkaline sol.	0.640 milligrams
No. 5 Flask containing live ferment in neutral sol.	0.860 milligrams

These nitrogen determinations show that in the alkaline fluid the proteid nitrogen during 26 days' incubation had increased about 25 times; in the neutral fluid over 33 times. These, of course, are values which are far beyond any possible source of error.

The records of the last described experiments show that when fresh or preserved saliva is incubated with nutrient fluids J50 and M35, a considerable rise of amylolytic power may occur which may persist for a shorter or longer period. Ptyalin evidently does much better in fluid M35 than in J50. It has also been shown that when saliva is mixed and incubated with these nutrient fluids the proteid nitrogen will increase from a mere, generally unmeasurable, trace to a considerable multiple of the original minute amount. There is, as far as we know, only one way in which nitrogen from simpler compounds such as are present in our solutions can be changed into proteid nitrogen—in other words, in which proteids can be formed or created—namely from proteins preexisting in the form of live matter; *hence there must be present something in our saliva-nutrient fluid mixtures that possesses all of the properties of life; that can perform the functions of nutrition, metabolism and reproduction; that can build up proteid nitrogen; and that unknown something which is present must be the live, multiplying, ultramicroscopic ferment granule, the true elementary organism, the ultimate organic biophoric unit.*

There appears to be no possibility of any other explanation of the results of the above experiments.

On the Increase, Decrease and Final Loss of Amylolytic Power in Some of the Nutrient Fluids

A number of experiments made with amylolytic ferments in the nutrient fluids illustrate important points about the increase, decrease, and final disappearance of the amylolytic power.

Experiment No. XVIII, September 23, 1917. Saliva

5 c. c. of a preserved saliva mixture, containing 2.5 c. c. of saliva, were added in a 250 c. c. volumetric flask to 245 c. c. nutrient fluid formula J35. (Formula J35 is identical with Formula J50, except that it contains 35 volume per cent of glycerine, instead of 50%).

Value of original amylolytic test	record lost
Value after 51 hours' incubation	277.9 milligrs. maltose
Value after 68 hours' incubation	308.6 milligrs. maltose
Value after 101 hours' incubation	299.6 milligrs. maltose
Value after 161 hours' incubation	288.4 milligrs. maltose
Value after 10 days' incubation	165.2 milligrs. maltose
Value after 13 days' incubation	163.1 milligrs. maltose
Value after 21 days' incubation	126.9 milligrs. maltose

FRESH HOGS' PANCREAS

Experiment No. XXV, Started December 20, 1917

About 5 grams of fresh hogs' pancreas were ground up in a meat grinder and dropped at once into 100 c. c. nutrient fluid, formula J50. The mixture was kept in the incubator for 22 hours, then filtered through filter paper, by the aid of a suction pump. A nitrogen determination of the filtrate shows 0.076 milligrams of proteid nitrogen per 1 c. c.

Ten c. c. of the filtrate were added in a volumetric flask to 240 c. c. nutrient fluid formula J50. The flask was then placed in the incubator at 37° C., December 21, noon.

Immediate amylolytic test was made as usual; added to the 40 c. c. of Phenol starch paste as described on page 18, 0.4 c. c. of the fresh filtrate plus 9.6 c. c. of nutrient fluid formula J50; the flask was kept for one hour in the water bath at 40° to 41° C.

Result of Amylolytic Test

In the subsequent tests, 10 c. c. from the 250 c. c. volumetric flask, containing 10 c. c. of the original filtrate plus 240 c. c. nutrient fluid were always added to the usual 1 gram starch paste.

Test made immediately with 0.4 c. c. of original filtrate	408.5 mgrs. maltose
Test made 20 hours after incubation	435.0 mgrs. maltose
Test made 44 hours after incubation	455.6 mgrs. maltose
Test made 68 hours after incubation	475.3 mgrs. maltose
Test made 92 hours after incubation	396.6 mgrs. maltose

Test made 116 hours after incubation	405.3 mgrs. maltose
Test made 212* hours after incubation	327.0 mgrs. maltose
Test made 19 days after incubation	261.9 mgrs. maltose
Test made 28 days after incubation	trace

Experiment with Acetone-Dry Hogs' Pancreas Powder

A number of experiments were made with an acetone-dry hogs' pancreas powder. This powder was prepared according to the method devised by Rapp for the preparation of an acetone-dry yeast powder for obtaining the enzyme zymase in a dry, comparatively stable form. The powder was prepared as follows: A number of fresh hogs' pancreas were weighed and then ground up fine in a meat grinder. The ground material was at once dropped into a vessel containing for each 100 grams pancreas 600 c. c. of pure, water-free acetone; it was then stirred in the latter for about 10 minutes, then filtered on a "Nutsche" through hardened filter paper, and pressed out. The dry cake thus obtained was broken up and divided and stirred in a second fresh portion of 600 c. c. Acetone, the latter again filtered off, and the dry cake again broken up, now brought for 2 to 3 minutes into about 150 to 200 c. c. of sulphuric ether, the latter then filtered off on the "Nutsche." The broken up material was finally dried completely in a vacuum at the temperature of the incubator. This procedure was performed in a tubulated dessicator kept in the incubator and connected with a suction pump by a rubber hose, leaving the incubator through a hole in its wall. The material after its removal from the dessicator, was pounded up repeatedly and thoroughly in a sterile mortar and finally sieved through a very fine brass sieve, having 100 meshes to the square centimeter. This separates all shreds of connective tissue, small vessels, etc., from an ultimately obtained very fine powder. The latter, which is quite hygroscopic, was again dried in a vacuum, or sometimes in a drying oven at a temperature a little above 100° C., and was either kept for use in a dessicator or distributed into Keidel bulbs from which the air had been exhausted. A powder prepared in this manner from hogs' or dogs' pancreas has a very strong amylolytic power, and in it can also be demonstrated the presence of other enzymes. The powder in a vacuum in the Keidel bulbs was permanently kept in the refrigerator and appeared to retain well, under these conditions, its amylolytic power.

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*A Nitrogen determination made December 26th, 1917, after 212 hours of incubation, gave a value of proteid nitrogen of 0.052 mgrs. per equivalent of the original 1 c. c. of gland extract. Hence there was at this time no increase, but already a decrease of proteid nitrogen. However, this experiment was mainly made to show the increase in amylolytic power which occurs first, and which is followed by a progressive decrease to a final vanishing point. The nitrogen determination in this experiment is of no value.

Experiment No. XXIV, with Acetone-Dry-Hogs' Pancreas Sieved Powder, December 17, 1917

One hundred milligrams of the dry powder were dissolved in 10 c. c. physiologic salt solution, shaken well and filtered through filter paper, 2.5 of the filtrate, plus 247.5 nutrient fluid J50 kept in a 250 c. c. volumetric flask in the incubator. A control flask, 100 c. c., containing 1 c. c. of the powder filtrate plus 99.0 c. c. nutrient fluid formula J50 boiled at once, was also kept in the incubator.

One c. c. of the filtrate plus 9 c. c. nutrient fluid formula J50 added to 40 c. c. of the usual 1 gram 2% Phenol starch paste; kept in water bath for 2 hours at 40° C., formed 423.1 mgrs. maltose. Repetition of the above test with 10 c. c. each time taken out of 250 c. c. flask.

Original test showed amylolytic power	423.1 mgrs. maltose
Incubation 17 hrs. 25 min. 2 hrs. fermentation amyl. power	310.5 mgrs. maltose
Incubation 41 hrs. 25 min. 2 hrs. fermentation amyl. power	337.7 mgrs. maltose
Incubation 65 hrs. 25 min. 2 hrs. fermentation amyl. power	334.1 mgrs. maltose
Incubation 89 hrs. 25 min. 2 hrs. fermentation amyl. power	339.2 mgrs. maltose
Incubation 113*hrs. 25 min. 2 hrs. fermentation amyl. power	319.5 mgrs. maltose
Incubation 209 hrs. 25 min. 2 hrs. fermentation amyl. power	315.5 mgrs. maltose
Incubation 329 hrs. 25 min. 2 hrs. fermentation amyl. power	296.2 mgrs. maltose
Incubation 23 days 2 hrs. fermentation amyl. power	90.6 mgrs. maltose
Incubation 32 days 2 hrs. fermentation amyl. power	trace (?)

The difference in this case is too small, it is still within the possible sources of error, hence it is of no value. In this experiment there was never any increase in amylolytic power, but a decrease occurred, evidently from the start. After 32 days' incubation the amylolytic power had practically disappeared completely. The protein Nitrogen at the start was comparatively high in this experiment. This was due to the use of 100 milligrams of acetone dry pancreas powder, of which 25 mgrs. were added to 250 c. c. of the fluid in the flask.

LOSS AND DISAPPEARANCE IN AMYLOLYTIC POWER AND DESTRUCTION OF THE AMYLOLYTIC FERMENT

In the preceding and other experiments it has been demonstrated that in the incubation of small amounts of saliva and of other amylolytic nutrient fluid J50, after the rise of amylolytic power a fall sets in after a certain number of days. The decrease may in a few days, or in a longer period, progress to a final complete loss of amylolytic power. The author has been familiar with the phenomena of the disappearance of amylolytic power in various fluids, even in the complete absence of

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*After 113 hours' incubation proteid nitrogen determinations were made with the following result:

Boiled control proteid N per 1 c. c. = 0.06 mgrs.
After 113 hours' incubation proteid N per 1 c. c. = 0.069 mgrs.

microorganisms, for many years, and he had long ascribed it to the presence of proteolytic ferments such as pepsin and trypsin. It has been shown by Buchner and Hahn, and others who have worked with the sugar splitting, alcohol and CO_2 forming enzyme zymase of the saccharomycetes, that yeast endo-tryptase, a proteolytic enzyme present in yeast cells and in their expressed juice, will speedily destroy zymase.

Trypsin and pepsin, provided ptyalin is composed of live protoplasmic granules, must of course be composed of the same type of live enzyme organisms. All these enzymes very probably are bodies in the nature of proteid granules. Hence if a proteolytic ferment in a solution like our nutrient fluids should destroy ptyalin granules present we would expect either no change in the proteid nitrogen or even a decided increase. The following experiment gives some information in this respect.

Experiment No. XXIII (October 20, 1917)

0.25 c. c. of preserved saliva, originally collected September 15, 1917, was placed into each one of two 250 c. c. volumetric flasks. The contents of the flasks were then made up to exactly 250 c. c. with nutrient fluid formula J50. The contents of one flask were boiled at once, then both flasks were incubated at 37° to 38° C. At intervals 5 c. c. were removed from each flask and the proteid nitrogen present was determined.

These analyses were carried out by using Gulick's micro-method. The result of the experiment started October 20, 1917, was as follows:

Date:	Oct. 21	Oct. 23	Oct. 25	Oct. 27	Nov. 7
Control (boiled saliva)........	0	0	trace	0	trace
Main test (unboiled) per 1 c. c.	0.08 mgrs.	0.13 mgrs.	0.1 mgrs.	0.11 mgrs.	trace
Total	20.0 mgrs.	32.5 mgrs.	25.0 mgrs.	27.5 mgrs.	trace

The tests made showed no appreciable amount of proteid nitrogen at any time in the contents of the boiled control flask. Measurable amounts of proteid nitrogen in the other non-boiled fluid were found. On October 27, 1917, there was still present a total of 27.5 mgrs. of proteid nitrogen; on November 7, 1917; i. e., after 10 days, this amount had dwindled down to a mere trace.

The above experiment shows that the proteid nitrogen had disappeared from the solution in which it had first increased considerably in amount. We must assume that the proteids, by the intervention of some factor, had been split up to much simpler bodies which do not furnish, in the tests employed, any proteid nitrogen. Now we know that proteid material in nature by the action of bacteria and other organisms can be split up to very simple chemical compounds such as ammonia, ammoacids, carbon dioxide, hydrogen-sulphide, water, etc. Under the author's conception, such changes as these bacteria and other organisms can bring about can be accomplished only by the aid of definite enzymes. We have every reason to believe that higher organisms must likewise possess such enzymes. It, therefore, appears that saliva contains such enzymes and that these are responsible for the final disappearance of the proteid nitrogen. We also are compelled to draw another conclusion from the result of the experiment; namely, that such unknown enzymes which can bring about the complete dissolution of proteids into very simple chemical compounds are themselves not proteid in character, but must be much simpler in structure and do not themselves give the reactions for proteid nitrogen.

ULTRAMICROSCOPIC DEMONSTRATION OF THE ENZYME GRANULES

The minute enzyme granules can be demonstrated very satisfactorily by the ordinary dark-field illuminator. A drop from nutrient fluid M35 neutral over agar is placed on a clean slide, covered with a cover-glass, and the slide is then laid in the usual manner into a drop of immersion oil over the dark-field illuminator. Examination with higher powers shows innumerable small, crowded, luminous dots, with here and there among the minute points, larger, apparently spherical bodies, probably made up of conglomerations of the individual ferment granules. When nutrient fluid M35 neutral is thus examined by the ultramicroscopic method, one is struck at once by the limited, not at all extensive, and very sluggish Brownian molecular movement of the luminous points. This was ascribed to the fact that the small particles displaced in M35 neutral are in colloidal suspension in a fluid containing 35% glycerine, hence of considerable viscosity. This interpretation of the observation proved to be correct, because a dilution of the fluid M35 with water brought about a more lively Brownian movement, which could be further increased by mixing the nutrient fluid containing the ferment granules with water that had previously been heated. The simple variations of the viscosity and the temperature of the medium in which the small granules were contained in colloid dispersion, beautifully showed how the Brownian movement is a function of ultimate molecular movements in the suspending fluid medium and of molecules which might be contained in molecular solution in such a medium. (The nutrient fluid M35 neutral, aside from water and glycerine, contains a number of salts, etc., present in molecular solution.)

EXPERIMENT WITH ASEPTICALLY REMOVED DOG'S PANCREAS

Experiment No. XXVI (December 28, 1917)

Dog's pancreas removed under all aseptic precautions. About 5 grams ground up in sterile meat grinder; the material was at once dropped into 100 c. c. nutrient fluid formula J50. The flask was placed in incubator (37° C.) and frequently shaken by hand during the next 24 hours. At the end of this period the contents of the flask were filtered through filter paper by the aid of a suction pump. The flask containing the filtrate was then kept in the refrigerator for a number of days, before the experiment could be continued.

January 11, 1918, 1 c. c. of the above mentioned dog's pancreas extract, plus 9 c. c. of nutrient fluid formula J50, added to usual 1 gram 40 c. c. 2% Phenol starch paste; kept in water bath for 2½ hours. Maltose formed 340.7 mgrs.

January 11, 1918, 5 c. c. of fresh dog's pancreas extract were added to 45 c. c. nutrient fluid J50. The flask (A) at once heated to boiling. 25 c. c. of the same dog's pancreas extract were added to 225 c. c. of nutrient fluid J50 and the flask was placed in a continuous water bath at 41° to 42° C. It was subsequently placed in the incubator and there kept at 37° C. Amylolytic tests made from time to time gave the following results:

Jan. 11, original test with 1 c. c. extract etc., 2½ hrs.' ferm.	340.7 mgrs.
Jan. 12, after 15 hours in water bath, 2½ hrs.' ferm.	412.8 mgrs.
Jan. 13, after 1 day, 15 hrs.' incubation, 2½ hrs.' ferm.	432.6 mgrs.
Jan. 14, after 2 days, 15 hrs.' incubation, 2½ hrs.' ferm.	458.4 mgrs.
Jan. 15, after 3 days, 15 hrs.' incubation, 2½ hrs.' ferm.	463.8 mgrs.
Jan. 16, after 4 days, 15 hrs.' incubation, 2½ hrs.' ferm.	481.3 mgrs.
Jan. 23, after 11 days' incubation, 2½ hrs.' ferm.	527.4 mgrs.
Jan. 26, after 14 days' incubation, 2½ hrs.' ferm.	534.7 mgrs.

Determination of proteid nitrogen gave the following results:

Proteid Nitrogen in boiled control (Flask A) in 50 c. c.	3.40 mgrs.
Proteid Nitrogen in (Flask B) after 2 days, 15 hrs.' incubation, per 50 c. c. fluid	3.90 mgrs.
Proteid Nitrogen in (Flask B) after 3 days, 15 hrs.' incubation	4.60 mgrs.
Proteid Nitrogen in (Flask B) after 18 days' incubation	4.50 mgrs.

MICROSCOPIC DEMONSTRATION OF THE PRIMORDIAL ENZYME GRANULES

The exhibition of innumerable very small round granules in fluids containing ptyalin by staining methods, which generally in addition to an intense stain, included the preliminary use of a mordant or fixative, in itself is not a convincing proof of the existence of live enzyme granules or primordial bioplastic units. It could with propriety be said that all fluids in which the author exhibited such granules contained proteids and the granules exhibited were simply proteid granules. Indeed, that is what they are, but they are not ordinary chemical compounds in the common sense of the word; but live, primordial granules, the ultimate units of life of which every form and type of protoplasm is composed.

The proof, however, that they are such live granules is not furnished by their mere microscopic or ultra-microscopic propagation experiments described above.

The microscopic exhibition of the primordial enzyme granula can be accomplished by the use of the following material and stains. It was desirable to use a starting material as pure and unmixed with other elements as possible; hence the saliva extract of salivary glands or of pancreas was not desirable for direct use as a starting substance, but the following material was used:

20 c. c. of nutrient fluid M35 neutral agar in a 100 c. c. Erlenmeyer flask was melted in a water bath and cooled down to about 60° C., when 1 c. c. of freshly collected, shaken, and filtered saliva was added. The fluid agar was well shaken and the mixture then allowed to cool and solidify. The agar flask was then placed in the incubator (37° C.) where it was kept for 12 days. Then 50 c. c. nutrient fluid formula M35 neutral was poured into the flask, which was then returned to the incubator. 10 c. c. of the fluid were then removed from the flask at intervals and allowed to act for two hours in the water bath at 41° to 42° C. on the usual 1 gram starch pastes, in order to test the amylolytic power of the fluid.

10 c. c. after 24 hours, 2 hrs. action on 1 gram starch, maltose formed 68.3 mgrs.
10 c. c. after 48 hours, 2 hrs. action on 1 gram starch, maltose formed 132.8 mgrs.
10 c. c. after 72 hours, 2 hrs. action on 1 gram starch, maltose formed 133.2 mgrs.

Microscopic smears were then made from the remaining fluid over the agar in the flask. The smears were made in the usual careful manner, as we prepare bacterial smears from fluid culture media when we want to stain for flayella.

1. Slides washed in soap and water, warm water, alcohol, then heated over flame.
2. Smears made from nutrient fluid M35 with platinum loop on clean slides which were protected against dust from the air.
3. Prepared slides placed for several hours in incubator, then removed and placed in hot air drying oven for 30 to 60 minutes, temperature allowed to go up to 125° C. (sometimes even to 135° C.).
4. Bunge's Mordant, prepared some time ago, consisting of—
   75 c. c. saturated watery solution of tannic acid,
   25 c. c. 5% solution of liquor ferri sesquichlorati,
   10 c. c. saturated watery solution of alkaline fuchsin.

After adding a few drops of hydrogen peroxide, this is filtered into a beaker. In the latter the prepared heated slides were placed so that the loaded side was inclined downward so that no precipitate could fall on the smear itself. The mordant in the beaker was then heated to steaming.

5. Slides washed well in distilled water.
6. Slides stained for 5 minutes with warmed Carbol Gentian Violett.
7. Well washed in distilled water and dried.

The slides so treated showed a dense deposit of very minute, generally faintly violet stained granules, though here and there a more intensely stained round granule can be seen. The granules are just at the boundary line of visibility when looked at with a 1/12 oil immersion lens. They are probably invisible in the unstained condition, because no doubt the mordanting and the staining with the deposit of many molecules of the mordant and the stain upon the granules increases their size considerably.

The following additional fixing and staining methods were subsequently also employed:

1. Preparation of another agar culture: to 20-25 c. c. of melted and cooled M35 neutral agar were added 2 c. c. of a freshly collected, shaken and filtered saliva. Agar solidified. Flask placed in incubator (37° C.) for 2 days. 60 c. c. nutrient fluid M35 neutral added; reincubated for two to several days. 15 c. c. nutrient fluid M35 neutral removed; action on usual starch paste tested; amylolytic action and saccharification very strong.

2. 10 c. c. of nutrient fluid M35 neutral removed from agar flask, added to 10 c. c. of a saturated picric acid solution. This was done to "fix" the granules. The mixture was then placed in a collodion sac, tied to a funnel and suspended, through a rubber cork, in a filtering flask. Suction was then applied, and the fluid filtering through the collodion sac was several times replaced by sterile distilled water, until the yellow color of the picric acid had become very faint. About 10 c. c. of the fluid finally remaining in the collodion sac was then poured into a small beaker, which was placed over (not into) a water bath. After the contents of the small beaker had evaporated down to about one-half the beaker was placed in a dessicator over sulphuric acid and left until the fluid had dwindled down to a few drops. These, with a platinum loop, were spread to perfectly clean slides, and the latter were then dried in the drying oven, the temperature being allowed to go up to 130° C. The dry slides were then immersed (standing up) in a beaker filled with saturated, freshly filtered, watery solution of fuchsin (alkaline for bacterial stains). The staining solution was then heated over a small flame to near boiling. The slides were then removed, washed in water, dried and examined with the oil immersion lens.

Osmic Acid Fixation

10 c. c. of the fluid from the agar flask culture in nutrient fluid M35 neutral were mixed with 10 c. c. of a freshly prepared ½% Osmic Acid Solution. The mixture was placed in the incubator for 24 hours, and slides were then prepared from the mixture. The slides were fixed in the hot-air drying oven as above; were then washed in distilled water; and examined, both unstained and stained, with hot saturated watery solution of fuchsin and with hot carbol-gentian-violett solution.

Heidenhain's Iron Hematoxylin Method

For this staining method corrosive sublimate fixation was used by either one of two methods; viz., smears were made from the nutrient fluid and the slides when dry were fixed in a saturated watery solution of corrosive sublimate, or an amount of nutrient fluid from the agar flask was mixed with an equal amount of saturated watery solution of $HgCl_2$. The mixture was allowed to stand for one hour and smears were then made on slides. These and the slides made by the other method were then placed in the drying oven at 125° C. When dry, the mercury was washed out in Lugol's iodine-iodide of potash solution, then the slides were washed several times in distilled water, and then treated according to Heidenhain's iron-hematoxylin method. The slides were always stood upright in the mordanting and staining solutions to prevent the formation of any deposit on them. Steps in the preparation of these iron-hematoxylin stains:

1. Fixation in saturated watery solution of corrosive sublimate (see above).
2. Heating in dry air oven up to 125° C. for 30 to 60 minutes.
3. Mordanting in a 2½% watery solution of iron sulphate of ammonium 8 hours.
4. Washing in distilled water.
5. Staining in ½% watery hematoxylin solution for 24 hours.
6. Washing in distilled water.
7. Slight differentiating in the iron sulphate of ammonium solution.
8. Washing in water, drying, etc. Examination with the oil immersion lens.

The four staining methods used all showed the same exceedingly small granules, equal in size and arrangement, either in very minute, discrete, point-like dots, surrounded sometimes by a refractive halo; or occasionally in double, diplococcus-like forms, or in small chains, or smaller or larger congregations of masses of granules. In some of these conglomerations the individual granules could still be distinctly seen—in others they were too densely crowded to permit of a resolution into individual granules.

DOES SALIVA CONTAIN A HORMONE OR AN ACTIVATOR FOR PTYALIN?

After the continued failures to transplant successfully and indefinitely from generation to generation, the enzyme granules which had undoubtedly much multiplied in the first generation in the nutrient media, as described above, the question arose whether salivary and other ptyalin perhaps acts only in the presence of a hormone or an activator; like, for instance, secretin, or like the activator of zymase, first described by Hardy and Young, and subsequently by others. Such hormones or activators, according to Starling, who introduced the name hormone (from the Greek root "hormao," I excite), have a comparatively simple chemical or molecular constitution and low molecular weight, are dialyzable and heat-resisting (thermostabile) but easily destroyed by oxidation.

In order to ascertain whether such a dialyzable hormone is present in saliva, the following experiment was made:

EXPERIMENT NO. XLII (MARCH 7, 1918)

This experiment was made with a collodion sac arranged for ultrafiltration under pressure as utilized in certain experiments in colloid chemistry.* A collodion sac in this experiment is tied to the stem of a funnel, the latter by means of a perforated rubber stopper fits tightly into a filtering flask which can be connected with a water suction pump. To 10 c. c. freshly collected, shaken saliva, filtered through filter paper, was added 10 c. c. of a 2½% NaFl solution. The mixture was then poured through the funnel into the collodion sac and the apparatus connected with the water suction pump. The fluid under pressure in the collodion sac came through the wall of the latter very slowly, only about 2 c. c. in one hour.

After half of the fluid had been dialyzed or filtered out of the sac, 10 c. c. of a 1.25% NaFl solution were poured through the funnel into the sac. This was, after intervals of several hours, repeated from time to time in order to remove all dialyzable or filterable substances through the wall of the sac and retain only the enzyme ptyalin with possibly present other non-dialyzable bodies. After 72 hours and after the contents of the collodion sac had been reduced to about 3 c. c. and after enough fluid had passed through the wall of the sac to leave only about one two-thousandth part of the original dialyzable substances contained in the 10 c. c. saliva used, the sac was carefully lifted out of the filtering flask, its closed end was split over a small conical graduate where the 3 c. c. were collected, the sac was then washed out with a 1.25% NaFl solution and the fluid in the graduate was made up to 20 c. c. The dialyzed and dilute saliva was then used in 5 c. c. lots on the usual 1 gram 40 c. c. 2% Phenol Starch pastes. The first 75 c. c. of fluid which had filtered through the collodion sac had been removed from the filtering flask and had been preserved in the refrigerator to be used in the experiment. The following tests were then made:

Each flask contained the usual (1 gram 40 c. c. 2%) Phenol starch pastes: Time of fermentation, 14 hours; temperature, 37° C. In the last flask (No. 7) the filtrate was allowed to act upon starch for 2½ days.

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*Wolfgang, Oswald: An Introduction to Theoretical and Applied Colloid Chemistry, translated by Martin M. Fischer, Wiley & Sons, New York, 1917, p. 48.

Flask No.	Dialyzed Saliva	2½% NaFl Sol.	Filtrate	Result after 14 hours at 37° C. Maltose formed
1.	5 c. c. plus	10 c. c. plus	0 c. c.	518.5
2.	0 c. c. plus	5 c. c. plus	10 c. c.	none
3.	5 c. c. plus	0 c. c. plus	10 c. c.	655.3
4.	5 c. c. plus	0 c. c. plus	10 c. c. (boiled)	663.7
5.	5 c. c. plus	8 c. c. plus	2 c. c. (boiled) saliva, but no filtrate)	678.4
6.	*boiled saliva 2 c. c. plus	13 c. c. plus	0 c. c.	(small, but marked trace.
7.	0 c. c. plus	0 c. c. plus	15 c. c. (left in incubator 2½ days)	none

The above experiment shows that salivary ptyalin does not contain a body in the nature of a filterable, heat-stable, hormone or an activator necessary for amylolytic action, because the thoroughly dialyzed saliva showed a very strong amylolytic action; however, it appears that the filtrate, both boiled and not boiled, and boiled saliva somewhat stimulated amylolytic action and produced an increase in the yield of maltose. The collodion sacs evidently hold back all of the ptyalin, because the filtrate alone showed no amylolytic effect, even after having acted on starch for 2½ days at 37° C. Even in spite of the above results, however, there might exist a hormone which influences the formation of ptyalin in the salivary glands; its existence is not disproven by the dialyzing experiment.

Propagation experiments were made with both fluid and solid gelatine and agar culture media in which saliva that had been heated in the steam sterilizer a sufficient length of time to destroy the ptyalin was added to the media to see whether it would supply any substance needed for progressive, unlimited propagation. These experiments were negative.

Proteid Nitrogen in Saliva

For mixed saliva the chemical formula given as to various constituents is generally as follows per 100 parts:

Total solids	0.5-1.0 per cent
Inorganic solids	0.4-0.6 per cent
Organic solids	0.1-0.4 per cent

The latter include mucin, serum albumin, and serum globulin, all of which will give the proteid nitrogen reactions.

The saliva used in our experiments was generally collected by four persons, then mixed, shaken in the shaking machine, then filtered through

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*The boiled saliva used in this experiment came from a lot of about 40 c. c. which had been brought to boiling over an open flame and yet as this and subsequent experiments showed, still contained some ptyalin which had not been destroyed.

filter paper, and preserved by the addition of an exactly equal amount of 1% Phenol-glycerine. Five specimens of such mixed, preserved, and filtered saliva were tested on February 11 and 12 for its amylolytic power and for the amount of proteid nitrogen it contained; the results of the tests were as follows:

Amylolytic tests on 40 c.c. 1 gram 2% Phenol Starch plus 5 c. c. of preserved mixed saliva, cont. 2.5 c. c. mixed saliva for 1 hour 30 minutes

Date when saliva was collected	Amylolytic tests made after collection	Maltose formed	Amylolytic tests made after collection	Maltose formed	Proteid Nitrogen Determination	
					Days after collection	Proteid N. found
					per 100c. c. saliva	
No. 1 10-1-17	133 days	472.4 mgrs.	146 days	328.0 mgrs.	146 days	40 mgrs.
No. 2 11-27-17	76 days	505.0 mgrs.	86 days	423.1 mgrs.	86 days	48 mgrs.
No. 3 1-24-18	20 days	523.8 mgrs.	33 days	no result	33 days	60 mgrs.
No. 4 2-11-18	0 days	525.8 mgrs.	13 days	461.6 mgrs.	13 days	92 mgrs.
No. 5 2-19-18	5 days	5 c. c. un-preserved pure saliva	5 days	583.3 mgrs.	5 days	95 mgrs.

Another proteid nitrogen test was made, not by a micro-method, but by Kjeldahl's method. 100 c. c. freshly collected, shaken, and filtered saliva were evaporated down to dryness in a beaker, kept over a water bath. The proteid nitrogen found by the Kjeldahl method was 84.3 milligrams; this would be equal to about 526.875 milligrams of proteid in saliva (factor of multiplication of N = 6.25). This figure is considerably higher than that for the organic solids generally given.

It appears from the above table that the amylolytic power of the preserved saliva kept in the refrigerator decreases with age and that simultaneously the amount of proteid nitrogen decreases likewise.

SALIVARY PTYALIN CONSIDERABLY MORE RESISTANT TO HIGHER TEMPERATURES THAN GENERALLY KNOWN

Statements found in literature concerning temperatures, absolutely destructive to enzymes, including ptyalin and diastase, are not trustworthy. Tests employed to show the complete destruction of the amylolytic and other enzymes have been deficient and unreliable, because the action of the enzymes after exposure to higher temperatures could not be permitted to go on long enough; viz., for days and weeks, to find out whether complete destruction had indeed occurred or had been simulated only by a temporary suspension of enzymatic action. The reason why such tests could not be prolonged as we do extend them, both culturally and by animal inoculations, after acting with higher temperatures upon bacteria and their spores, was that we heretofore had no means to test enzymatic action for a prolonged period under truly and perfectly aseptic conditions with the exclusion of every possible bacterial contamination. This fact is strongly emphasized by Effront,* who states: "To summarize, we do not possess a sure and practical means of keeping a solution of trypsin protected from all infection for a length of time surpassing 6 to 10 hours. If we use large quantities of antiseptic, we run a great risk of weakening or destroying the enzyme; if we are content with small doses, we get no preservative action."

Our nutrient fluids containing from 35 to 50 volume per cent of glycerine, and in addition 2% Phenol or 2½% NaFl enable us to study the action of amylolytic enzymes for periods of days and weeks without the least danger of bacterial contamination.

The following experiments were made to find out the temperature and time of complete destruction of ptyalin.

EXPERIMENT No. XXXIX (FEBRUARY 28, 1918)

Saliva had been collected, shaken, and filtered as usual, on February 19, 1918, and had continually been preserved without any addition, in the refrigerator. Test made to find its amylolytic power on February 28, 1918. 1 c. c. saliva, plus 9 c. c. nutrient fluid formula M35 neutral added to usual Phenol-starch paste. Fermentation allowed to go on in the incubator (37° C.) for 24 hours. Maltose formed 490.3 mgrs. The same saliva was then kept in the water bath for 30 minutes at 56° to 57° C. Then 1 c. c. of the heated saliva plus 9 c. c. nutrient fluid formula M35 neutral were allowed to act upon starch as above for 24 hours. Maltose formed, 302.0 mgrs. This shows that 56° to 57° C. acting during 30 minutes has a weakening effect, but not a decidedly destructive action.

EXPERIMENT No. XL (MARCH 1, 1918)

Freshly collected, shaken, and filtered saliva was placed in a water bath at 56° to 57° C., and 1 c. c. of the heated saliva was taken out at intervals and added to the usual starch paste plus 9 c. c. of the nutrient fluid formula M35 neutral.

Test No., time, heated at 56° to 57° C.	Fermentation in incubator at 37° C.	Maltose formed
No. 1 not heated	4 hours	647.1 mgrs.
No. 2 30 minutes	4 hours	632.2 mgrs.
No. 3 40 minutes	4 hours	639.0 mgrs.
No. 4 50 minutes	4 hours	616.0 mgrs.
No. 5 60 minutes	4 hours	542.3 mgrs.
No. 6 80 minutes	4 hours	509.9 mgrs.
No. 7 100 minutes	4 hours	538.4 mgrs.
No. 8 120 minutes	4 hours	450.3 mgrs.

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*Jean Effront: Biochemical Catalysts, etc., transl. by Prescott & Venable. John Wiley & Sons, New York, 1917, p. 312.

No. 9 140 minutes	4 hours	425.4 mgrs.
No. 10 160 minutes	4 hours	72.0 mgrs.
No. 11 210 minutes	4 hours	starch becomes soluble, still trace of maltose
No. 12 240 minutes	4 hours	Same as No. 11
No. 13 300 minutes	15 hours	Same as Nos. 11 and 12

The above series shows that ptyaline is first not much affected by being exposed to a temperature of 56° to 57° C. It can stand this temperature for over 2 hours; there comes a time, however, when its power of resistance against this higher temperature vanishes; the ptyalin molecule, perhaps after having been compelled to undergo the larger vibrations equivalent to the elevated temperature of 56° to 57° C., for a certain length of time, goes to pieces and is transformed into another chemical molecule. The crisis evidently comes rather suddenly, because the loss of amylolytic power after heating for 140 minutes is rather moderate, while it becomes very large after heating for 160 minutes. The destructive effect after from 160 to 300 minutes' heating at 56° to 57° C. can hardly be looked upon as a damage referable to coagulation.

EXPERIMENT NO. XLI (MARCH 2, 1918)

Experiment similar in arrangement to the preceding one, except that the fresh, filtered saliva was kept in the water bath at 70° C., and that the fermentation starch flasks were kept in the incubator for longer periods of time.

Test No. heated at 70° C.	Fermentation in incubator at 37° C.	Maltose formed
No. 1 immediate test	5 hours	716.5
No. 2 5 minutes	5 hours	205.3

(The contents of the flask containing the saliva were warmed to 40° C., then the flask was placed in the large water bath; within 2 minutes the temperature of the saliva was up to 70° C., so that the fluid was really exposed 3 minutes to 70° C. when the amylolytic test was made.)

No. 3 10 minutes	5 hours	No maltose
No. 4 15 minutes	13 days	Maltose—small amount
No. 5 20 minutes	13 days	Maltose—small amount
No. 6 25 minutes	13 days	Maltose—small amount
No. 7 30 minutes	13 days	Maltose—small amount
No. 8 40 minutes	13 days	Maltose—small amount
No. 9 50 minutes	13 days	Maltose about 40 mgrs.
No. 10 60 minutes	13 days	Maltose trace
Control flask kept in incubator	13 days	Maltose None

From the above series it appears that 70° C. is a decidedly damaging temperature for salivary ptyalin. It damages the enzyme considerably after a few minutes' action (3 to 5 minutes). After acting 10 minutes it apparently destroys ptyalin, but this is not indeed true, the action of ptyalin is only suspended, but it can be shown not to have been destroyed completely if the amylolytic fermentation is allowed to go on for days.

EXPERIMENT No. XLIV (MARCH 15, 1918)

This experiment is similar to the preceding ones, except that the temperature of exposure of the saliva was most of the time from 80° to 81° C., and the water bath finally went up to 82° C.

Test No. heated at 80° to 81° C.	Fermentation in incubator at 37° C.	Maltose formed
No. 1 immediate test	2 hours	452.4 mgrs.
No. 2 5 minutes	11 days	250.0 mgrs.
No. 3 20 minutes	11 days	55.0 mgrs.
No. 4 40 minutes	11 days	51.0 mgrs.
No. 5 60 minutes	11 days	small amount
No. 6 80 minutes	11 days	small amount
No. 7 100 minutes	11 days	small amount
No. 8 130 minutes	11 days	trace

This table shows that heating at 80° to 82° C. for 130 minutes did not completely destroy ptyalin, but left a trace of enzyme which could form a trace of maltose from starch in eleven days.

In the following two experiments the temperature of boiling water, as it prevails at the Chicago elevation above sea level was allowed to act upon saliva.

EXPERIMENT No. XLV

About 40 c. c. fresh, filtered saliva in a 100 c. c. Erlenmeyer flask was heated over a medium-sized flame of a Bunsen burner. As soon as boiling occurred the flask was removed from the flame and allowed to cool spontaneously. Subsequently 20 c. c. of this boiled saliva were added to the usual Phenol-starch paste, plus 20 c. c. nutrient fluid M35 neutral. This flask was kept in the incubator for 13 days. Maltose formed, 72 milligrams.

EXPERIMENT No. XLIII (MARCH 11, 1918)

75 c. c. fresh, filtered saliva in an Erlenmeyer flask, warmed up to 40° C., were placed in an Arnold steam sterilizer which had steam up, for an hour. From time to time 2 c. c. of the heated saliva were removed and added to starch pastes plus 8 c. c. M35 neutral.

Test No. Kept in steam sterilizer	Fermentation in incubator at 37° C.	Maltose formed
No. 1 0 minutes	1 hour	295.0 mgrs.
No. 2 10 minutes	13 days	68.8 mgrs.
No. 3 20 minutes	13 days	marked trace
No. 4 30 minutes	13 days	marked trace
No. 5 45 minutes	13 days	none

No. 6 60 minutes	13 days	none
No. 7 60 minutes	13 days	*

It is well to call attention to the fact that when the amylolytic enzyme has been greatly damaged by a sufficiently long exposure to higher temperatures, it may appear to be completely destroyed for several days and no effect upon starch pastes is manifest. After several days, however, a slow, limited saccharification appears, which, however, does not become extensive at all and leads, no matter how long one waits, to the formation of only small amounts of maltose.

PROPAGATION EXPERIMENTS

After it had been shown that ptyalin derived from various sources when incubated with the nutrient fluids described above (J35, J50, M35 and M50) shows an increase of amylolytic power and an increase of proteid nitrogen, it was considered as fully proven that the enzyme was not an ordinary chemical compound but a live body composed of minute ultimate bioplastic primordial granules which possess all the metabolic and propagating properties of life. It appeared that it should now be easy, with the aid of the nutrient fluids devised, to propagate from generation to generation without any limit; yet this hope was not fulfilled; there are evidently present obstacles which have not been conquered and the nature of which is not yet fully understood.

The following are some examples of the numerous experiments of transplantation undertaken:

Experiment No. XXXVIII (February 27, 1918)

Test-tubes were prepared, each one containing 10 c. c. of nutrient fluid M35 neutral. Test-tube No. 1 was inoculated 3 times with a platinum loop dipped into freshly collected, shaken, and filtered saliva. The tube was then incubated at 37° C. for two days. Then a tube No. 2 was inoculated, 3 platinum loopfuls from No. 1 and so forth. After a number of successively inoculated tubes had been kept in the incubator for a number of days, the contents of the inoculated tubes were poured over to 100 c. c. flasks containing the usual Phenol-starch paste. These flasks were then incubated for 2 days and their contents were then examined for maltose. Result of the examination:

Flask No. 1 from test-tube No. 1.............353.2 mgrs. maltose
Flask No. 2 from test-tube No. 2................trace of maltose
Flask No. 3 and all others and control.......no maltose whatever

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*In this test, No. 7, 20 c. c. of the heated saliva plus 20 c. c. M35 neutral were added to the starch paste and the flask remained in the incubator for 13 days. It was then found to contain a marked trace of maltose; hence it appears that even after 1 hour's stay in the steam sterilizer, 20 c. c. of saliva still contained a trace of active, not completely destroyed, ptyalin.

After numerous failures in fluid media, solid media, viz., gelatine and agar, were tried. With agar an interesting observation, fully described below, was made.

FAILURE OF SALIVARY PTYALIN TO INCREASE WHEN TRANSPLANTED FROM INOCULATED NUTRIENT FLUID M35 TO THE SAME MEDIUM

Experiment No. XXXI (January 20, 1918)

Original inoculation Dec. 13, 1917, 2.5 c. c. fresh, shaken, and filtered saliva plus 247.5 c. c. nutrient fluid formula M35 incubated. Original amylolytic power Dec. 13, 1917, after 39 days had risen to 339.2 mgrs. On January 20; i. e., 38 days after incubation 10 c. c. of the developing nutrient mixture was added to a fresh portion of 240 c. c. M35 which had been completely neutralized by the addition of H_3PO_4. The mixture was placed in the incubator but its amylolytic power was never very high, and it decreased, so that the experiment was discontinued. The values obtained were as follows:

	Maltose formed
Jan. 21, after 1 day's incubation—ferm. starch in incub. 6 days	231.9 mgrs.
Jan. 27, after 7 days' incubation—ferm. starch in incub. 1 day	150.1 mgrs.
Jan. 28, after 8 days' incubation—ferm. starch in incub. 1 day	92.8 mgrs.
Feb. 2, after 12 days' incubation—ferm. starch in incub. 1 day	163.9 mgrs.
Feb. 3, after 13 days' incubation—ferm. starch in incub. 1 day	191.1 mgrs.
Feb. 7, after 17 days' incubation—ferm. starch in incub. 1 day	187.3 mgrs.
Feb. 13, after 23 days' incubation—ferm. starch in incub. 1 day	134.8 mgrs.

The same experiment had previously been made with nutrient fluids J35 and J50, and the flasks containing the starch pastes plus the nutrient fluid from the test-tubes were kept in the incubator in some instances for several weeks. The result was always the same. Abundant maltose formed in flask No. 1; but only a trace in No. 2 and sometimes in No. 3. All other tubes up to 10 and more showed no maltose.

PROPAGATION EXPERIMENTS IN GELATINE

Experiment No. XXX (January 19, 1918)

A nutrient gelatine had been prepared as follows: To nutrient fluid, formula M35, alkaline, was added 20% of French gelatine as commonly used in bacteriologic work. The gelatine was dissolved in the steam sterilizer (or over the open flame) and was then, without being filtered, distributed to 100 c. c. Erlenmeyer flasks, about 20 to 25 c. c. to each flask. The culture material was then sterilized by fractional sterilization in the steam sterilizer. When inoculated, the gelatine was melted in hot water at about 40° to 45° C., then there was added to the melted gelatine a definite amount of fluid containing an active amylolytic ferment. The flask, after the addition of the latter, was well shaken and then rapidly cooled in cold water or in the refrigerator. It was then kept for 7 or 8 days at room temperature.

The flasks inoculated with gelatine may be exposed to diffuse daylight; this does not interfere with the outcome of the experiment. After 7 to 8 days the gelatine was melted and at the same time the gelatine in a flask No. 2 was also melted. About 15 to 20 drops were then poured over from flask No. 1 to flask No. 2. The latter was rapidly cooled; while to the No. 1 flask the usual Phenol-starch (warmed to about 40° C.) was added, the flask was then well shaken, and it was placed in the incubator (37° to 40° C.) where it was left for a number of days and shaken several times each day. After No. 2 had been kept for 7 or 8 days at room temperature it was treated like No. 1, and so forth. All flasks remained in the incubator and the progressing saccharification could be observed from day to day. When starch is acted upon by the amylolytic enzymes, it becomes first liquefied; and later on a more or less clear transparent layer forms on top, while a finely flocculent material sinks to the bottom. In the Experiment No. XXII the amylolytic material used for the first flask was 5 c. c. of a 2.5 c. c. saliva-247.5 c. c. nutrient fluid (M35 alkaline) mixture started Dec. 13, 1917. This fluid (see Exp. No. XXII) had shown a great increase of amylolytic power and an increase of proteid nitrogen on January 18, 1918. Hence 5 c. c. of the nutrient mixture was added to flask No. 1, M35 alkaline gelatine on Jan. 19, 1918.

Result of Above Culture Experiment with Human Salivary Ptyalin

Flask No.	Date and source of inoculation	Date when placed in incubator	No. of days in incubator	Maltose formed
1.	Jan. 19, 1918, saliva plus nutrient M35 alkaline	Jan. 19, 1918	47	310.5 mgrs.
2.	Jan. 26, 1918, from flask No. 1	Feb. 2, 1918	40	56.6 mgrs.
3.	Feb. 2, 1918, from flask No. 2	Feb. 9, 1918	33	trace
4.	Feb. 9, 1918, from flask No. 3	Feb. 16. 1918	26	none
5.	Feb. 16, 1918, from flask No. 4	Feb. 23, 1918	19	none
6.	Feb. 23, 1918, from flask No. 5	Mar. 2, 1918	12	none
7.	Mar. 2, 1918, from flask No. 6	Mar. 9, 1918	5	none
	Controls..		variable number of days	none

CULTURE EXPERIMENT IN GELATINE WITH ACETONE-DRY POWDER FROM HOGS' PANCREAS

EXPERIMENT No. XXVIII (JANUARY 1, 1918)

The inoculated material was derived from an acetone-dry powder of hogs' pancreas. A small amount of a suspension of this powder* in physiologic salt solution had been mixed with nutrient fluid J50, had been incubated from Dec. 17 to Dec. 31, 1917. Its amylolytic power, after 14 days, had decreased from 423.1 mgrs. to 296.2 mgrs. There had never been an increase; yet this nutrient fluid, as the following experiment shows, contained ptyalin granules, able to multiply from generation to generation in the J50 gelatine. The gelatine used was prepared as described above; however, the nutrient fluid used was J50 plus 20% dissolved gelatine, distributed in flasks about 20 to 25 c. c. to each. Flask No. 1 on Jan. 1, 1918, received 5 c. c. of the acetone-dry powder of hogs' pancreas—nutrient fluid J50† mixture. The procedure of inoculation and transplants was exactly the same as in the preceding experiments.

Flask No.	Date and source of inoculation	Date when placed in incubator	No. of days in incubator	Maltose formed
1.	Jan. 1, 1918, from acetone-dry-hogs' pancreas, and nutrient fluid J50	Jan. 8, 1918	1	147.5 mgrs.
2.	Jan. 8, 1918, from flask No. 1	Jan. 15, 1918	2	41.9 mgrs.
3.	Jan. 15, 1918, from flask No. 2	Jan. 22, 1918	50	about 1.5 mgrs.
4.	Jan. 22, 1918, from flask No. 3	Jan. 30, 1918	42	trace (?)
5.	Jan. 30, 1918, from flask No. 4	Feb. 6, 1918	35	none
6.	Feb. 6, 1918, from flask No. 5	Feb. 13, 1918	28	none
7.	Feb. 13, 1918, from flask No. 6	Feb. 21, 1918	20	none
8.	Feb. 21, 1918, from flask No. 7	Feb. 28, 1918	13	none

EXPERIMENT No. XXVII (DECEMBER 28, 1917-JANUARY 20, 1918)

With fresh dogs' pancreas extract. 5 grams freshly ground up, aseptically obtained dogs' pancreas dropped into 100 c. c. nutrient fluid formula J50. Kept in incubator (37° C.) until Jan. 20, 1918. 5 c. c. of the original extract on Jan. 20, 1918, were added to 20 to 25 c. c. melted J50 gelatine. Flask kept at room temperature until Jan. 28, 1918. Contents melted in water bath, 1 to 2 c. c. transferred to flask No. 2 containing the same J50 gelatine. To flask 1, the usual Phenol-starch paste added. Flask placed in incubator. Result of tests for maltose formed:

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*100 mgrs. of dry powder dissolved in 10 c. c. physiologic salt solution, filtered, 2.5 c. c. of filtrate to 247.5 c. c. nutrient fluid formula J50.

†While using formula J50 in this and other experiments, I could not get any more ammonium lactate in the American market, so I had to substitute for the ammonium lactate, asparagin in 1 gram amounts per 1000 c. c. nutrient fluid. The new nutrient fluid J50 did as well as the old formula with ammonium lactate.

Flask No. 1 incubated with starch paste 6 days—maltose formed	716.3 mgrs.
Flask No. 2 incubated with starch paste 37 days—maltose formed	230.5 mgrs.
Flask No. 3 incubated with starch paste 30 days—maltose formed	35.0 mgrs.
Flask No. 4 incubated with starch paste 22 days—maltose formed	trace (?)
Flask No. 5 incubated with starch paste 15 days—maltose formed	none
Other flasks ....maltose formed	none

TRANSPLANTATION IN AGAR CULTURE MEDIA

Experiment No. XXXIII (February 1)

Experiments to transplant from generation to generation in agar were made in the following manner: A 1½% agar was prepared with nutrient fluid M35 neutralized with phosphoric acid. The agar was not filtered but cleared by sedimentation, the medium was allowed to cool slowly in a large beaker in the steam-sterilizer, and after solidification the lowest layer of the mass, caked out of the receptacle, was cut off and thrown away. The re-melted clear agar was re-melted and then distributed to 100 c. c. Erlenmeyer flasks, 20 to 25 c. c. to each one. In the first inoculation 1.0 c. c. of a preserved saliva was added to an agar flask, the contents of which had been melted and cooled down to about 50° C. After the addition of the saliva the contents of the flask were rapidly cooled by immersing the latter in cold water. The inoculated flask was then placed in the incubator. After 2 days it was taken out and the agar in another flask (No. 2) was melted and cooled down to 50° C. Then a piece of the agar in flask No. 1 was cut out with a steel-hook,* sterilized over the flame, and the cut out piece of agar was dropped into the melted agar of flask No. 2. Its contents were then rapidly cooled and the flask, after solidification of its contents, was placed in the incubator. After flask No. 2 thus had been inoculated from flask No. 1 the latter received 40 c. c. of the usual Phenol-starch paste. Flask No. 1 was then replaced in the incubator. After the lapse of 2 days flask No. 3 was inoculated from No. 2 and so forth. Result of transplants:

	Maltose formed
No. 1 incubated 2 days plus starch paste—reincubated 2 days..........	445.1 mgrs.
No. 2 incubated 2 days plus starch paste—reincubated 9 days..........	281.0 mgrs.
No. 3 incubated 2 days plus starch paste—reincubated 7 days..........	30.0 mgrs.
No. 4 incubated 3 days plus starch paste—reincubated 7 days..........	0(None)

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*When flask No. 3 was inoculated from flask No. 2, pain was taken to cut out from flask No. 2 a piece of agar distant from the place to which the piece from flask No. 1 had been fastened when the agar in flask No. 2 solidified.

EXPERIMENT NO. XXXVI (FEBRUARY 19, 1918)

5 c. c. of saliva-nutrient fluid M35 neutral which had been incubated from Jan. 23 to Feb. 19 were added to melted saliva, the subsequent steps as in the preceding experiment:

	Maltose formed
Flask No. 1 incubated 1 day plus starch paste, reincubated 1 day....	226.5 mgrs.
Flask No. 2 incubated 2 days plus starch paste, reincubated 2 days...	175.0 mgrs.
Flask No. 3 incubated 2 days plus starch paste, reincubated 3 days...	37.6 mgrs.
Flask No. 4 incubated 2 days plus starch paste, reincubated 8 days...	trace
Flask No. 5 incubated 2 days plus starch paste, reincubated 6 days...	none
Flask No. 6 incubated 2 days plus starch paste, reincubated 4 days...	none

The above experiment shows that maltose was formed abundantly only in flask No. 1 and rapidly decreased from flask to flask.

Amylolytic Enzyme Reforming from Day to Day in Agar

The following experiments show that the amylolytic ferment may repeatedly be extracted from the agar by starch pastes. As the agar successively softens, however, the yield of amylolytic ferment becomes less and less, and finally dwindles down to zero.

EXPERIMENT NO. XXXIV (FEBRUARY 7, 1918)

Agar flask plus 1 c. c. preserved saliva; incubated for 24 hours plus the usual Phenol-starch paste. Reincubated for 18 hours; saccharified starch paste was then poured off and the maltose determined; the agar flask then received a new lot of Phenol-starch paste, and the flask was again incubated and the saccharified contents examined after 6 hours. The following results were obtained:

Starch addition No. 1 incubated 18 hours, yield in maltose............	468.1 mgrs.
Starch addition No. 2 incubated 6 hours, yield in maltose............	463.9 mgrs.
Starch addition No. 3 incubated 24 hours, yield in maltose............	437.5 mgrs.
Starch addition No. 4 incubated 24 hours, yield in maltose............	396.9 mgrs.
Starch addition No. 5 incubated 24 hours, yield in maltose............	287.3 mgrs.
Starch addition No. 6 incubated 96 hours, yield in maltose............	77.8 mgrs.

Agar now completely softened.

EXPERIMENT NO. XXXVII (FEBRUARY 19, 1918)

Added to agar flask No. 1, 5 c. c. saliva-formula M35 neutral mixture which had been incubated 27 days. In this experiment the agar softened more rapidly and the yield in maltose came down rapidly.

Starch addition No. 1 incubated 24 hours, yield in maltose............	226.5 mgrs.
Starch addition No. 2 incubated 24 hours, yield in maltose............	175.0 mgrs.
Starch addition No. 3 incubated 72 hours, yield in maltose............	37.6 mgrs.

Agar now completely softened.

EXPOSURE OF PTYALIN TO FREE HCl

Crittenden and *Smith* have stated that the presence of 0.03 per cent of free HCl will speedily destroy ptyalin completely. This statement is only apparently correct. It is true that under the conditions of the experiments as arranged by the investigators the amylolytic power of ptyalin appears destroyed, yet it can be shown that it is not all immediately gone, but that it can be brought back. The following experiment illustrates this point. The acidulation was made in the same manner.

Experiment No. XXIX (January 3, 1918)

20 c. c. of fresh, shaken, and subsequently filtered saliva were neutralized (litmus) with 0.2% HCl, about 75 c. c. distilled water were added, then 1 c. c. of a standardized 3% solution of HCl was added, and the whole made up in a volumetric flask to exactly 100 c. c. The dilute saliva then contained per 100 c. c.—0.03 free HCl. The mixture was then kept for 30 minutes in a water bath at 39° C., it was then taken out and neutralized to litmus with a 5% solution of Disodic Hydrogen Phosphate. It took about 16 c. c. of this solution to produce complete neutralization. When this dilute, acidulated (0.03% HCl), and then neutralized saliva was directly tested on starch pastes, it showed no amylolytic power. The dilute saliva was then treated as follows: 35 c. c. of it were added to 140 c. c. nutrient fluid J50 and the flask was then incubated (37° C.). The following tests were then made by taking 20 c. c. out of the flask and having it act on the Phenol-starch paste generally used:

	Maltose formed
16 hrs. after incubation 20 c. c. to 1 gram starch paste ferm. 24 hrs. about	50.0 mgrs.
4 days after incubation 20 c. c. to 1 gram starch paste ferm. 25 days about	93.0 mgrs.
18 days after incubation 20 c. c. to 1 gram starch paste ferm. 11 days about	39.6 mgrs.
24 days after incubation 20 c. c. to 1 gram starch paste ferm 4 days about	38.1 mgrs.
26 days after incubation 20 c. c. to 1 gram starch paste ferm. 2 days about	35.0 mgrs.
30 days after incubation 20 c. c. to 1 gram starch paste ferm. 9 days about	62.1 mgrs.
37 days after incubation 20 c. c. to 1 gram starch paste ferm. 4 days	no more maltose formed, but paste still liquefied

Experiment No. XXXV (February 17, 1918)

20 c. c. of fresh filtered saliva were acidulated, as in the preceding experiment. To 65 c. c. of the dilute, heated, and subsequently neutralized saliva were added 35 c. c. of pure neutral glycerine. Then 25 c. c. of this dilute-saliva-glycerine mixture were added to 125 c. c. nutrient fluid formula M35 neutral. The flask containing this 150 c. c. mixture was then incubated at 37° C., after 20 c. c. of its contents had been removed and had been added to the usual Phenol-starch paste. The

latter was then kept in the incubator for 24 hours. No maltose was formed. 20 c. c. of the contents of the flask containing the dilute-saliva-nutrient fluid M35 neutral were removed from time to time and tested on starch pastes. Results of these tests:

24 hrs. after incubation 20 c. c. to 1 gram starch paste ferm. 24 hrs...35.0 mgrs.
48 hrs. after incubation 20 c. c. to 1 gram starch paste ferm. 24 hrs...73.5 mgrs.
72 hrs. after incubation 20 c. c. to 1 gram starch paste ferm. 192 hrs...65.0 mgrs.
312 hrs. after incubation 20 c. c. to 1 gram starch paste ferm. 96 hrs...82.2 mgrs.

After the dilute-saliva-nutrient mixture had been incubated 2 days, 5 c. c. were removed from the flask and poured into a flask containing 20 c. c. of a melted M35 neutral agar. After solidification of the agar the flask was placed in the incubator for 4 days, then the usual Phenol-starch paste was added and the flask was reincubated for 7 days. 40 mgrs. of maltose were formed.

THE SURVIVAL OF GRANULES BEST FITTED TO RESIST HCl

We have demonstrated by our experiments that a percentage of 0.03 free HCl does not completely destroy all of the amylolytic enzyme of saliva exposed, but that it only inactivates ptyalin partially and that a part of the enzyme at least survives and can be reactivated after neutralization and after being placed, under the proper condition, into nutrient fluids. This is a very important observation, because it shows that under natural conditions some of the amylolytic ferment granules, mixed with food and brought to the stomach will after neutralization in the duodenum be in a condition to be reactivated, provided that they are placed under favorable conditions. There can be no doubt that a proteid material like ptyalin will be easily absorbed from the small intestines and will enter the general blood and lymph circulation. These granules with the lymph and blood stream will then be brought to all of the tissues of the body, but they will speedily perish except in one place, namely, in the cells of the salivary glands from which they originally came. Here they find, of course, the most favorable condition for their metabolism and reproduction, because they are right back to their normal habitat. But why should they in their invasion with the blood and lymph into all other cells perish everywhere except in the cells of the salivary glands? It is obvious that every cell of a multicellular being will tolerate in its interior only ferment granules which naturally belong there, because any permanent invasion of a cell by foreign ferment granules would completely disturb its metabolism and endanger its very existence. Of course, invasions by foreign ferment granules do occur for a longer period, or even permanently, but we are then dealing with pathologic processes such as cloudy swelling, fatty, caseous, and other degenerations, or finally with complete neurosis and cell death. Ultimately cellular pathology is granular pathology. But we do not want to go into any pathologic processes now, but want to see whether a re-entrance of ptyalin enzyme granules from the small intestines into the salivary gland cell, their reawakening there, followed by reproduction, would be of any importance from a general biologic standpoint.

It would be of the greatest importance, and in fact, what we know of the laws of variability, evolution, and inheritance imperatively demand such possibilities of the re-entrance of ferment granules to their normal habitat after they have been exposed to outside influences in their specific activity. Amylolytic ferment granules found in various organisms must, of course, be all the descendants of a common ancestor and they must have been modified and must have found varieties according to the requirement of the plant or animal of which they form an integral part. If we take a higher animal where the ptyalin granules are located and are reproduced in glands, their modification would only be possible very indirectly and very slowly unless granules that had done their work in the stomach could re-enter the salivary glands. There would practically, even under changing conditions, be very little, if any, chance of modification during the lifetime of an individual, unless re-entrance into the glands can occur.

Now let us see what may happen if there is a chance for re-entrance into the gland cell. Let us suppose that an individual suffers from a strong hyperacidity of the gastric juice. Salivary ptyalin entering the stomach, everything else being equal, will have the less chance to act for a period of time in the stomach, the more free HCl is present, and at the same time a lesser number of live ptyalin granules will arrive in the small intestines, will have a chance to be reactivated and to re-enter the salivary gland cells. But all those ptyalin granules which do enter the gland cells, there to recover, will have the property of being more resistant to a higher percentage of HCl than the average ptyalin granule. It is immaterial to inquire into the question "To what property is it due that one ptyalin granule survives and another does not in the presence of certain percentages of free HCl?" That this is the case was undoubtedly shown by our experiments, because a large percentage of the ptyalin granules undoubtedly are killed in the presence of 0.03 HCl, but some few survive and can again be made to do amylolytic work. Such differences in behavior undoubtedly are due to individual variability. That the live ptyalin granules possess this characteristic was also shown by the experiment in which a strongly alkaline nutrient fluid was used (see exp. XXXV and remarks). The re-entered ferment granules would, of course, impart to their descendants those properties which enabled them to withstand higher amounts of HCl than they are exposed to under perfectly normal conditions. Thus there would, after a number of generations, be produced a race of ptyalin ferment granules more resistant to HCl than the original stock. That this would be of advantage to an individual suffering from gastric hyperacidity is obvious. The modified generations of ferment corpuscles would also be supplied in the course of time to the maturing sex cells, and thus the modified character acquired during a certain term of years would also be handed down from the ancestor to the offspring. In the case of the live amylolytic enzyme granules we have considered an isolated possible case, but the same conclusions must be reached in every possible case with every other elementary organism of live beings. But each individual case will, of course, present conditions which must be considered as to the probable modus operandi.

THE NATURE OF TOXINS AND THE FALLACIES OF EHRLICH'S SIDE-CHAIN THEORY

The experiments made with the amylolytic ferment ptyalin have shown that an enzyme is not, as heretofore believed, a chemical compound in the ordinary sense of the word, but a very minute live elementary organism. The experiments made with tetanus toxin have shown that the latter is not a chemical compound in the ordinary sense of the word, but an ultramicroscopic, filterable live virus. It must be indeed a very dull intellect that would not be able to see at once that the enzyme ptyalin and the toxin of the tetanus bacillus are elementary organisms of the same type and order. If that is true, as a matter of course, all enzymes, toxins, antitoxins, antigens and antibodies must be live elementary organisms and not merely dead chemical compounds. But how can we explain the action of antigens and antibodies, in particular that of the tetanus toxin and the tetanus antitoxin, if we look upon them as enzymes; i. e., live bioplastic units, and how does Ehrlich's side-chain theory fare from our standpoint?

Let us consider what views we have been able to form from our experimental work with ptyalin. This enzyme goes through a cycle of changes of generation, every generation has its peculiar fermentative products. One generation multiplies in the gland cells—of its metabolic products we know nothing. The next generation brings about that change in starch which makes it soluble, which perhaps is identical with the production of amylodextrin. The next generation from amylodextrin forms erythrodextrin; the next one from the latter achroodextrin. The next one from the latter—maltose.

Whether there is only one definite chemical compound or whether there are several erythrodextrins and each one is attacked by a different generation of the ferment granules, does not require any discussion now, since it is immaterial for our present considerations.

Now let us suppose that ptyalin entered the blood circulation and that we have also ptyalin, enzyme starch present in the blood current. The granules first multiply in the blood and they produce in their metabolism unknown products which are not poisonous. Then the granules change generation and they now produce from starch amyloerythro—and achroodextrin. These are all violent poisons belonging to an identical chemical group, or they may be poisons somewhat different in their effect, somewhat like tetanospasmin and tenanolysin. Finally if the invaded organism survives in spite of the action of the strong successively forming poisons all of the dextrins are changed into maltose by the last generation of our ferment—elementary organism. Maltose is not a poison, hence the invaded organism recovers from the effect of the invasion. Now let us suppose that the last generation of the ferment organism, which changes the poison dextrin into the harmless substance maltose, could survive sometime in the blood current of the invaded host, and could be removed with the blood-serum and injected into another host. If the latter then would be invaded by the first generation of ptyalin granules, dextrins as soon as formed would be converted into maltose by the host of granules of the last generation present. No slow accumulation of dextrins could occur, hence a poisoning could not take place. In our hypothetical, ultramicroscopic, filterable virus, ptyalin the first generation, would therefore act like the tetanus toxin, the last generation like the tetanus antitoxin, and we could use our virus ptyalin just as we use our tetanus toxin, to produce passive and active immunization.

There is another analogy between our hypothetical ptyalin-toxin and our tetanus toxin. It has been shown by Calmette and Wassermann in several instances of toxins and their antitoxins that the latter are less resistant to unfavorable influences than the former. Temperatures which will destroy an antitoxin will in several instances not destroy its toxin. In our experiments with the enzyme ptyalin we have again and again seen that such unfavorable influences as acid, antiseptics, and higher temperatures will so change the constitution of the elementary organisms that only the first and not the last generations can be formed. Hence we still get liquefaction of the starch and dextrin formation even when maltose is not formed.

It is clear from our above considerations that a foreign elementary organism transported by microorganisms or otherwise into a higher organism can produce disease only if it can multiply, go through certain changes of generation, and produce harmful metabolic products. That disease will terminate either by—

(a) Stoppage of process with elimination of poisonous products; that is, by excretion or secretion.
(b) Change of generation of organism with change of poisonous products into harmless.
(c) Stoppage of process due to congenital characteristics.
    (1) The Elementary organisms may not multiply and perish at once, or
    (2) They may multiply, but the organism may possess enzymes which act at once upon the poisonous product.

What Ehrlich has called a toxin-spectrum is also due to a number of generations of disease producing enzyme granules and to their changing metabolic products. That toxin and antitoxin clearly entertain to each other quantitative relations can also be seen from a consideration of the action of the enzyme granules. Suppose there are present 1000 individuals of a generation which produce as their metabolic product, the poison, then we would need 1000 individuals of the next generation to change at once all of the poison produced to the harmless substance. If there are only 500 of the last generation present there remains an excess of the poison which will only be changed gradually by a change of generation of the poison-producing generation.

According to the views of the side-chain theory all anti-bodies against the antigens are produced by the body cells of the invaded organism in the following manner, a side-chain of the antigen attacks a side-chain of the cell, destroys it, and causes an over-production of the destroyed side-chains. The overproduced side-chains are thrown off from the cell, get into the circulation in the blood serum, and appear as the various antibodies, such as antitoxins, agglutinins, precipitous lysins and hemolysins.

It is very remarkable that Ehrlich's side-chain theory never has been attacked seriously from a general biologic standpoint, and that it has never been pointed out that the existence of the numerous cell side-chains which the theory requires could not be comprehended or explained under the general laws of evolution. We inject into the circulation of a rabbit, sheep, or human blood-corpuscles, and then according to the side-chain theory, the cells of the rabbit possess and form in excess specific chemical side-chains, which act only upon certain side-chains of the sheep or human blood-corpuscle. Now it never happens under natural conditions that heterogeneous blood-corpuscles are injected into the circulation of an animal. How, therefore, in the course of evolution could the rabbit acquire a complicated apparatus which under natural conditions never is called upon to perform a specific function? We already know a very large number of antibodies against antigens of various types. It is strange that nobody has yet pointed out that according to the side-chain theory the organism, especially its cells, possesses almost innumerable biochemical apparatuses which are called into function in the artificial experiment only, and perhaps never under natural conditions. Heterogeneous proteids of an almost endless variety are taken into the gastro-intestinal tract and are there acted upon by ferments and changed. But why should the cells of the organisms possess a great number of specific side-chains to form specific antibodies against a large number of various heterologous proteins which in reality are never introduced by a par-enteral route, except experimentally?

How then could we, for instance, explain the formation of a hemolysin in the body of the rabbit, specific only for sheep's corpuscles? It can be explained in the following manner: In reporting the result of experiments with ptyalin we have called attention to the fact that this enzyme, under certain experimental conditions, is destroyed so that it loses its character as a proteid body and is split up into simpler compounds. The destruction of the enzyme ptyalin was referred to the action of unknown protein splitting enzymes which decompose the former into final very simple chemical compounds. We know that all cells of multicellular beings have a certain term of existence and then age and degenerate frequently by lytic processes; that is, by processes of solution brought about by enzymes. Every cell, it appears, contains in its own interior these enzymes of final dissolution. The red blood-corpuscles certainly contain such lytic enzymes, because red blood-corpuscles undergo hemolysis under normal and numerous pathologic conditions. It is evident that whenever a red blood-corpuscle is damaged by bacterial, thermic, chemical, and other influences, it is dissolved; and we have every reason to believe that the hemolysins exist in the red blood-corpuscles themselves. Now when we inject the blood-corpuscles of a sheep into the circulation of the rabbit, the sheep's corpuscle is placed under very unfavorable environments, and it must undergo pathologic changes; i. e., probably lytic changes, due to its own hemolysins. The latter will dissolve the sheep's corpuscle, set free the hemoglobin, and then hemolysin from the sheep's corpuscles will get into the serum of the rabbit and there exist for some time as the being it is; namely, a live ultimate primordial (hemolytic) granule. If we then draw the treated rabbit's blood we have in it a hemolysin specific for sheep's corpuscles, but this specific sheep's corpuscle hemolysin has not been formed by the side-chains of the rabbit's cells, but has been furnished and supplied by the sheep's erythrocyte where it has its normal habitat and where its first generation multiplies when the proper conditions are created in the body of the sheep as also in the body of the rabbit. What we have called complement and amboceptor are not separate bodies in the old sense of the term, but they are alternative generations of the same primordial granulum, which in its various changes brings about various molecular changes in the substances acted upon. When we draw the blood of a rabbit which has been injected with sheep's corpuscles, the serum contains that generation of hemolysin granules which follows the first proliferating generation. The first specifically active hemolytic generation brings about a certain change in the hemoglobin and undergoes a certain change itself. In other words, it has changed into amboceptor, and hemolysis can now occur. If we allow the fresh hemolytic rabbit's serum to stand for some time, or if we treat it for 30 minutes to 56° C. we change the complement generation into the amboceptor generation. This, of course, cannot start hemolysis, just as we could not get maltose by acting on starch paste with the last generation of ptyalin granules. What we call complement fixation by an antigen—antibody action must be an effect upon a complement by an antigen—antibody which causes the complement to change generation so that it can no more effect the first chemical change in consequence of which the so-called amboceptor change cannot be initiated.

CONCLUSION

The decrease in maltose on the addition of maltose may indicate that maltose is a product of metabolism; hence the natural outcome is that the living granules perish or cease to be active in their own metabolic products.

The increase of maltose in the enrichment media can be explained in only one of two ways which are as follows:

1. Nutrient formula J50 represents a mixture in which the amylolytic ferment can be well kept for a number of days, and in this mixture the zymogen of ptyalin develops rather gradually but with fair rapidity into the active enzyme; hence what the author has been doing is to develop in his so-called nutrient fluid a heretofore more or less unknown zymogen of human saliva into the active enzyme.
2. The second interpretation, which we will endeavor to prove, is that ptyalin is not an unorganized chemical compound, but is a live ultramicroscopic ferment granule, an elementary organism, and it assimilates, grows, and multiplies or propagates, not only under natural conditions in the salivary glands, but also in the author's artificial culture medium, formula J50.

The increase in nitrogen in the various experiments is by far the most interesting and valuable data of the whole paper. While in many cases the increase in nitrogen was not great, in most of the experiments the increase was marked and can lead to only one conclusion, providing there was no error in the technique of analysis. This objection is scarcely fair, however, for the analyst had performed almost a thousand such determinations previously. The samples for analysis were prepared, labeled, and given to the analyst without his knowing the contents, the analyses made, and the results returned as they are given in this paper. The only conclusion, therefore, that can be drawn from these experiments is that, in that protein-free medium containing a trace of fresh saliva there developed from the various chemicals present a nitrogeneous substance which was coagulable, similar to protein compounds, and that there was no development in the sample that was similarly prepared, but boiled. This coagulable proteid, or whatever you wish to call it, was beyond doubt increased or reproduced from the trace of living ptyalin granules which were inoculated into the medium.

We can readily see that the life cycle is almost if not quite complete; we have apparent metabolism; we have growth and reproduction; we have death. In short, we have a substance that possesses all the properties of life, that can grow and reproduce; that can build up proteid nitrogen; and that can perform all of the necessary functions of life. This substance must be the ultramicroscopic ferment granules—the true, ultimate, organic life-bearing units.

Assuming then, that we have found that ferments are living bodies, it is interesting to speculate the possible role of ferments in the system of evolution of life. If all life originated from some ultimate unit, it must have been some such bodies as ferments that were these units, the first life of the universe; the first matter that migrated out of the realm of the inert and passed over into the kind of matter that is able to grow and reproduce. Such fantasy, of course, is beyond the scope of this work. We give it as fantasy and let it be accepted accordingly.

It will do no harm, however, to complete the picture of evolution. The beginning of energy is as much of a mystery as ever. From energy we have positive and negative charges; from these we have atoms, molecules, and the beginnings of worlds, nebulæ, spiral nebulæ, solar systems; first forms of life, the simplest collection of protein molecules, the heat-resisting ferments; then the ultramicroscopic organisms, bacteria, and from there begins our own evolution system so well known by all.

The heat-resisting ferments were possibly incorporated into greater living units as time has passed. Hence we have the diastatic ferment that is scarcely impaired at 60° C.

PART II

* * * * mais pourquoi ne dirais-je pas, que j'aie cette confiance intime que les action des ferments solubles s'effaceront un jour même dans la physiologie des êtres supérieurs devant celles de la vie sans air.—Pasteur.

GENERAL INTRODUCTORY REMARKS

If one views critically what has been heretofore taught about the nature of the enzymes, the so-called soluble, unorganized ferments, one is compelled to confess that those bodies, non-organized as they have been supposed to be, are chemical compounds which do not follow the elementary laws of physics and chemistry, bodies which are not bound and regulated in their action by the laws of the conservation and correlation of energy as laid down by Robert Mayer, Helmholtz and Joule.

I will endeavor to show in this article, by a series of experiments conducted on a chemical and mainly on a bacteriological basis, that malt diastase and ptyalin are by no means unorganized dead bodies, but consist of exceedingly small round granules, which grow and multiply in the proper nutritive soils, not only in the grain of malt and in the alveolar secretory cells of the salivary glands, but also in artificial soils, kept in the incubator, and which form, in the presence of starch, dextrins and sugar as products of their metabolism.

It has been taught heretofore that the action of the enzymes in general and the amylolytic action of diastase and ptyalin in special is to be looked upon as a catalytic process.

By catalysis, contact, or cylical action, is to be understood a class of chemical changes that are induced in certain chemical compounds or agents by a substance which does not itself undergo any permanent alteration, but whose presence under suitable conditions brings about a rearrangement among the molecules of the bodies with which it is placed in contact. The material acting in this manner without apparently being affected itself by the changes it induces, has been termed a catalytic or contact agent.

We know a large number of catalytic processes, the purely nonanimated chemical nature of which is beyond any doubt at all. The conversion of saccharose (cane-sugar) by dilute acids into invert sugar (glucose lævulose); the conversion of the glucosids by dilute acids into glucose and another body or bodies, depending upon the special glucosid; the process of converting starch into dextrin and sugar by dilute acids at high temperatures are examples of such catalytic processes.

In the process of converting starch into dextrins and sugar through the medium of the catalytic action of dilute acids a great number of caloric units are needed to bring about this chemical change. In other words, we have a conversion of kinetic energy (heat) into potential energy which is stored up in the products (dextrin and sugar) of this catalytic process.

On the other hand, if we convert starch into dextrins and sugar by the action of an amylolytic ferment latent heat becomes free or we have a conversion of potential energy into kinetic energy.

That this is really the case in the conversion of starch by the amylolytic ferments may be shown by the following simple experiment:

A fresh active malt extract is to be prepared. This extract is filtered through an ordinary paper filter and 200 c. c. of it are placed in a flask and put into the thermostat. Another flask of a capacity of about 500 c. c. containing 200 c. c. of a 2% starch paste is likewise placed in the thermostat, thermometers are to be passed through openings of the incubator into the two flasks.

When the temperature in both flasks has become constant and equal, the thermostat is opened and the malt extract is poured over into the flask containing the starch paste. Saccharification begins at once and the temperature in the flask containing the fermenting mixture rises in a very few minutes several (2°-3° C.) degrees.

In both instances of converting starch—conversion by dilute acids and conversion by amylolytic ferments—the final products are the same, i. e., dextrins and sugar; they must, therefore, represent the same values of potential energies, yet in the former case heat becomes latent, in the latter case latent heat becomes free, in addition to effecting the same chemical change which in the former case requires the expenditure of so great an amount of kinetic energy. This certainly shows that the action of amylolytic ferment is very different from the true catalytic action of dilute acids. The former (diastatic ferment action) can only be brought into concert with the laws of the correlation and conservation of energy if we assume that in the totality of the changes brought about by these enzymes, there must be, aside from the conversion of the starch into dextrins and sugar, some additional process which has been entirely overlooked heretofore and which represents a change from potential energy to kinetic energy.

Urech[2] has studied the catalytic process of the conversion of saccharose by dilute acids into invert sugar. He has found by a number of carefully made experiments that the amounts of invert-sugar formed are equal in equal periods of time (of course everything else being equal in the experiment). So if we shall designate a number of periods of time of equal intervals with t_1 t_2 t_3 t_n and the amounts of invert-sugar formed in one interval equal to m, we get for t_1 t_2 t_3 t_n minutes, amounts of invert-sugar equal to m, 2m, 3m, nm. A curve illustrating graphically the law of the formation of invert-sugar in this catalytic process would be a straight line, rising from 0 to $nm = 100$ (complete inversion of all the saccharose) in t_n minutes. Urech also states particularly that the amount of invert-sugar formed has no hindering influence upon the continuation of the process of inversion.

There are quite a number of contradictory statements on record concerning certain details about the amylolytic ferments, yet all observers and experimenters are unanimous in the statement that the formation and accumulation of sugar in a starch solution undergoing diastatic fermentation acts as a hindering agent to the continuation of the process of conversion.

I could not find in going over the literature of the subject that anybody having studied the diastatic ferments has tried to define the hindering influence of maltose upon a fermenting starch solution by adding a certain percentage of maltose from the start. I have, therefore, made a series of experiments under these conditions.

The maltose was prepared according to a plan given in the new part, see p. 8, exp. A.

Of the solution so obtained a few c. c. were taken, diluted, and the amount of maltose contained was ascertained by several titrations. The medium of the results was taken as the indicator of the percentage of maltose in the solution prepared. This solution was then diluted to make it up to a 20% watery solution of maltose. From it solutions of varying strength could be conveniently prepared.

To 40 c. c. of maltose solutions of varying concentration one gram of cornstarch was next added and the flasks containing the mixtures were heated in a water bath under continual shaking in order to secure a homogeneous starch paste. After the contents of the flasks had cooled down to 46° C., 10 c. c. of a 10% saliva-sterile-distilled-water mixture was added to each one. In this and in all following experiments, when it was necessary to measure off exact equal quantities the saliva was always diluted with 9 or 4 times its volume of sterile distilled water.

After the 10 c. c. of the dilute saliva had been added the fermentation was allowed to go on for two hours. It was then brought to a stop by heating the flasks and their contents for some time in a steam sterilizer.

The amounts of maltose formed in addition to those previously added at the beginning of the experiment were ascertained by titration, as follows:

Maltose in solution from the start	Maltose formed in addition
0%	0.811
1%	0.783
2%	0.453
3%	0.270
4%	0.210
5%	0.066
6%	0.031
7%	0.032
8%	traces
9%	traces
10%	traces

These series of experiments demonstrate clearly the correctness of observation that the accumulation of the maltose formed in the conversion of the starch by the amylolytic ferments does act as a hindering agent.

In the catalytic process of the conversion of saccharose into invert-sugar by dilute acids the formation of the invert-sugar has, as ascertained by Urech, no hindering influence upon the continuation of the process. If the conversion of starch by the amylolytic ferments were a process analagous to the former we ought to expect that the crops of sugar formed in equal intervals of time should diminish in a certain proportion on account of the increasing accumulation of the hindering fermentation product.

If we assume as constant, as has been done heretofore, the mass of the fermentative agent (the enzyme) and if we consider what we cannot deny and what nobody has denied, that the increasing formation of maltose acts as a hindering agent of an increasing proportion, or, expressed mathematically, as a variable of values increasing according to a definite law, we must expect that the amounts of maltose formed in equal periods of time should decrease in a regular proportion. Reduced to a mathematical formula the amounts of maltose formed in a starch solution undergoing diastatic fermentation after the lapse of equal periods of time ought to be represented by the formula—

$$\overset{t_1}{m} \qquad \overset{t_2}{2m - x_1} \qquad \overset{t_3}{3m - x_2} \qquad \overset{t_4}{4m - x_3} \qquad \overset{t_n}{nm - x_{n-1}}$$

This, however, is not the case. On the contrary, all observers who have investigated this point have found that there is at first a great increase in the proportion of the amounts of maltose formed in equal periods of time.

The following series of experiments, which I have made, demonstrates the above proven fact, stated unanimously by all observers.

Action of 1 c. c. of saliva upon 100 c. c. of a 2% starch solution in water containing small percentages of chloride of sodium, peptones, glycerine, and diphosphate of sodium, for increasing periods of time.

Experiment B

No. 1 Maltose formed after 2 minutes............0.160 grams
No. 2 Maltose formed after 4 minutes............0.300 grams
No. 3 Maltose formed after 6 minutes............1.059 grams
No. 4 Maltose formed after 8 minutes............1.210 grams
No. 5 Maltose formed after 10 minutes............1.244 grams
No. 6 Maltose formed after 12 minutes.......... bottle broken
No. 7 Maltose formed after 14 minutes............1.344 grams
No. 8 Maltose formed after 16 minutes............1.545 grams
No. 9 Maltose formed after 18 minutes............1.569 grams
No. 10 Maltose formed after 20 minutes............1.584 grams

How could such behavior of the amylolytic ferment be explained in view of the hindering influence of the maltose formed unless the amount of ferment did increase during its action and at a rate which in the beginning of action is so great as greatly to overcome the hindering influence of the maltose formed?

A REVIEW OF THE LITERATURE ON DIASTASE AND PTYALIN

A good many contradictory statements are yet to be found in literature with reference to a number of points concerning the amylolytic action of diastase and ptyalin. Even about the final product of fermentation; i. e., maltose, there are faulty statements to be found in publications of recent date. Unfortunately, an observer of merit years ago came to a wrong conclusion which has been disproven since then by many, but which still appears in many textbooks.

W. D. Miller[5] in his excellent book on the microorganisms of the buccal cavity, for instance, says: "Ptyalin is distinguished from diastase in the first instance by a difference in the product of fermentation. It forms from starch ptyalose-sugar, while malt diastase forms maltose; in the second instance ptyalin has its optimum of action at a temperature of 35° to 40° C. while malt diastase acts best at a temperature of 70° C." Both statements are incorrect. Both ferments form as the product of their fermentative action maltose; the optimum temperature of ptyalin is higher and that of malt diastase is lower than the figures given by Miller. It is indeed a fact that 70°, far from being the optimum temperature of malt diastase, is one, which, if acting long enough, will destroy diastase. Kjeldahl[6] by a long series of experiments has ascertained an optimum temperature of 63° C. for malt diastase, yet the increase in the amount of sugar at temperatures varying from 50° to 63° C. is very slight.

At temperatures higher than 63° the yield in sugar is rapidly diminished until it is very little at 70°; while, as my experiments show, malt diastase will form no sugar at all from starch if previously kept long enough at a temperature of 70° C. The optimum temperature for ptyalin, according to Kjeldahl as well as according to my own experiments given in detail below, is 46° C. Before Kjeldahl published his extensive article on diastase, O'Sullivan,[7] as the result of a series of experiments, came to the conclusion that temperatures above 63° are inimical to the amylolytic end product of the diastatic fermentation. He finds that at temperatures below 63° C. the crop of sugar and dextrin is as 67.85% to 32.15%; at temperatures ranging between 64° to 68° C. the crop of sugar and dextrin is as 34.54% to 65.46%; and at temperatures ranging between 68° to 70° C. like 17.4% (maltose) to 82.6% (dextrin). O'Sullivan is very much impressed by this observation and feels inclined to believe that diastase cannot be, according to these observations, a homogeneous body but something consisting of a number of different constituents. He, therefore, raises the question * * * * "or does malt extract contain three distinct transforming bodies, one decomposing starch according to equation A, destroyed at 64° to 70° C., and another as in equation B destroyed at 68° to 70° C., whilst a third causes decomposition according to equation C, becoming inactive at a point not yet determined. * * * * Hence it would appear that the varying decompositions are not due to any difference in the size of the starch molecule, but to some change brought about by heat in the decompositing agent or agents."

O'Sullivan does not attempt to answer this question which is forced upon him from the results of his experiments.

From these observations it appears that the optimum temperatures of malt diastase and ptyalin are not very far apart. The final product of the fermentative action of both upon starch and glycogen is, as will be seen below, identical; namely, maltose and isomaltose.

There is, therefore, no reason to believe in a generic difference between the vegetable malt diastase and the animal ptyalin cells, because a slight difference in the optimum temperatures alone cannot establish such a distinction. Dieudonne[8] has shown that microorganisms can be adapted in a comparatively short time to new optimum temperatures, to temperatures which previously were manifestly unfavorable to them. A long chain of phylogenetic development and evolution therefore certainly offers the possibility of an adaptation to differences in the optimum temperatures of metabolic changes.

Ptyalin has been found in the saliva of all domestic animals examined for it, also in the saliva of rabbits and guinea pigs. Before taking up in detail the experiments made in order to ascertain the true nature of diastase and ptyalin I would like to call attention to an error found in most textbooks. It is frequently stated in the books that starch becomes soluble in hot water. It is said that in the latter the starch cellulose bursts open and the starch granulose (the starch proper) now becomes soluble. If the latter is to be obtained pure, it is to be precipitated by alcohol, filtered, dried, and redissolved in water. I have prepared this solution according to this prescription, (1 pt. starch to 100 pts. dist. water); but when this alleged solution was examined under the microscope in the form of the hanging drop, the milky-white, translucent starch granules could be seen suspended in the fluid. One ought not, therefore, speak of a solution of starch in water, but of a starch paste, a starch suspension, or emulsion. Whenever the word starch solution is used in the following pages it is to be considered in its proper sense. Kirchhoff[9] was the first investigator who from his observations and experiments arrived at a fairly clear conception about the formation of sugar from starch in the grain of the cereals. In his communication concerning this subject published in the early part of this century, he criticises the older views of Cruikshank, Saussure, and Thompson, and others, and gives a resume of his own experiments, from which he draws, among others, the following conclusions: Gluten is the cause of the formation of sugar in the growing grain and also of this change in boiled starch. The starch is present as such in the grain, and at a temperature of 40° C. and over is converted into sugar by the gluten. The converting agent of the malt which Kirchhoff still called gluten, was first isolated by Payen[10] and Persoz.[11] In their first publication concerning their discovery they make the following introductory remarks: "Depuis les savants recherches et les travaux laborieux de Leuwenhook, Saussure, Kirchhoff, Vauquelin, des brasseurs anglais, de M. M. Dubrunfaut, Raspail, Guibourt, Converchel, etc., on connaissait la conformation physiologique de l'amidon * * * * * * qu'a l'aide de l'orge germic de l'eau, et de la chaleur la fecule etait saccharifiée." They finally report the sum and substance of their discovery as follows:

"Cette substance que nous sommes parvenus à isoler, contient d'autant moins d'azote, qu'elle approche plus de l'etat de pureté et possède d'ailleurs les proprietes suivantes: elle est solide, blanche, amorphe, insoluble dans l'alcool, soluble dans l'eau et l'alcool faible * * * chauffée de 65° to 75° avec de la fécule, elle presente le pouvoir remarquable de detacher promptment les enveloppes de la substance interieur modifiée, la dextrine, qui se dissont façilement dans l'eau tandis que les téguments insoluble dans ce liquide surnagent ou se precipitent, suivant les movements du liquide. Cette singulière proprieté de separation nous a determiné à donner à la substance qui la possède le nom de *diastase,* qui exprime précisement ce fait. L'orsque l'extraction de ce principe immediat nouveau a été faite avec soin, son energie est telle qu'une partie en poids suffit pour rendre soluble dans l'eau chaude la substance intérieur de deux milles parties de fécule sèche, et pour operer ensuite la conversion de la dextrine en sucre."

The first communication was soon followed by a number of others by the same authors, by Payen [12] alone, and by Guerin-Varry,[13] and in a few years a good deal was known concerning malt diastase. In later communications Payen[14-15] also reported his experiments, demonstrating that the action of diastase stopped by the formation of a certain maximum amount of sugar may again be made manifest on the removal of the sugar.

The conclusions of Payen concerning this point were later confirmed by Lindet[16] who showed that when maltose has accumulated to a certain percentage in a fermenting starch solution it hinders the continuation of the conversion of the starch. He showed that when the sugar formed has been removed the process of saccharification can continue. To Dubrunfaut[17] belongs the credit of first having discovered that by the action of malt diastase upon starch a special sugar, and not glucose as had been formerly believed, was formed. This fact was announced in 1847 and Dubrunfaut called the sugar formed by the action of malt diastase upon starch, maltose. He found that this sugar had triple the rotatory power of glucose, was less soluble in alcohol, and was converted into glucose by boiling with dilute mineral acids. Dubrunfaut's discoveries were not accepted, however, and were soon forgotten until O'Sullivan[18] not only confirmed them but also established them on such a firm basis that they are now universally accepted. O'Sullivan also discovered the fact that maltose in reductive power was very much inferior to glucose and he established the exact proportion of their respective reductive powers, 66 (maltose); 100 (glucose). It was a number of years after Kirchhoff had recognized the process of saccharification of amylum by a certain constituent of the grain of malt that the action of saliva upon starch received the proper attention of scientific investigators.

Saliva, preferably the human saliva, has played in former centuries a very prominent role in medical literature, because a number of miraculous therapeutic effects were ascribed to human saliva, especially with reference to diseases of the eyes. It is a mistake if recent literature assumes that the fermentative power of saliva was only recognized in the first half of our century. Wild tribes of the old, as well as of the new, world have for centuries made practical use of the amylolytic fermentative power of the human saliva in the preparation of their alcoholic beverages. They have mixed with amylaceous vegetable products saliva in vessels or have first chewed such substances and then expectorated this product of masseteric activity into vessels. This mass so prepared was left standing in the open air and in proper time saccharomyces getting into the fluid so treated with saliva preliminarily, did the rest in manufacturing alcoholic stimulants. These customs are reported in works of travelers as well as in books by medical writers. According to Baglivi,[19] who devotes to saliva a good deal of space in his works, the word saliva is derived from salt (sal) because it sometimes approaches in taste to salt or abounds in salt, or communicates its salt to the chycle and the blood, by which it promotes and excites the vital fermentations and preserves and cherishes life. Zwinger[20] accounts for its derivation from the same root, because salts of various kinds are separable from saliva by chemical resolution. According to others the word is derived from the Greek word σαλευω = salenó which signifies flowing as in a frothy sea.

Baglivi (l. c.) says about human saliva: "Inductor, ut credam salivam continere in se sal nitro-salinum universali sali analogum ob eximias, ac prorsus mirabilis satis veres, equas solvendo, fuendo, abstergendo, et licet insipida videatur, potentur, penetrando in fermentatione ciborum, purificazione chyli, ejusdem que in sanguinem mutatione concotionesque absolvit."

Boerhaave,[21] too, attributes to saliva a fermentative power, he even found that this power was exerted upon starch. He says: "Inutile futurum puto hic multis probare velle salivam liquore esse, abstergentem, solventem, et mire penetrantem, cum ipsius effectus apud vultus notissimi sint * * * * * *massam farinceam fermentat* * * * * * ut merito dici possit humani naturi sapo.

"In vulgus notum est, Indos potus suos inebriantes ita praeparare ut vetulae edulentulae, grana mays manducent, et succum cum saliva mistunt expuant in proprium vas terum, in quo moderato tempore excoquator in cerevissiam illis gentibus acceptissimam syrupi illapsa per incuriam saliva visi sunt acidi et turpidi facti esse."

The fermentative power of saliva was rediscovered and the product of the fermentation was recognized by Leuchs[22] who by experimental inquiry ascertained that saliva by its action upon starch formed sugar. Leuchs' discovery was soon confirmed by Schwann, Sebastian, and Wright. Others, however, denied without reserve that saliva did produce sugar by its action upon starch. Consequently, years after Leuchs had made his discovery we find the most varied views concerning the action of saliva. Claude Bernard and Barreswil[23] claimed (of course at a time when the former had not yet discovered glycogen and the glycogenic function of the liver) that saliva has no specific action at all. They say: "Le suc gastrique, le fluide pancréatique et la saliva renferme une même principe organique, actif dans la digestion, mais c'est seulement la nature de la réaction chimique, que fait différer la rôle physiologique de chacun de ses liquides et qui détermine leur aptitude digestif pour tel ou tel principe alimentaire."

Very soon the experiments by which Bernard and Barreswil arrived at this strange conclusion were shown to be fallacious by Jacubowitsch[24] and Frerichs. Still in 1852 the question as to the physiological character of the saliva is still "sub judice" and Lehmann[25] in his extensive work on "Physiological Chemistry" says: "The chemical function of the saliva is a subject on which different observers have held very different views. Leuchs was the first who was led by experimental inquiry to the discovery that starch is gradually converted into sugar by the action of saliva. Subsequent inquirers who have repeated the experiments in some instances confirm and in others deny the accuracy of his views." Finally Lehmann believes that he should deny any action of the saliva upon aliments, and in the "Additions and Notes" to his work, he says, on page 499, Vol. III: "We are consequently led by the earlier observations of Bernard as well as by the more recent investigations of Bidder and Schmidt, to the conclusion that notwithstanding its energetic action on starch, and notwithstanding its abundant supply, the saliva takes no very important part in the digestion of amylum." This view appeared ten years after the investigators named and two French physiologists, Mialhe and Magendie, had ascertained beyond a doubt the amylolytic power of the saliva. Mialhe[26] was the first investigator who claimed that the amylolytic action of the saliva was due to a certain constituent of this secretion. In 1845 he submitted the results of his researches to the French Academy of Sciences. His articles prove that he had indeed in a certain sense isolated ptyalin, that he ascribed to it the amylolytic action of saliva, and that he recognized influences inimical to this action. From his reports the following may be cited: "* * * * * J'ai donc cherché quels phenomènes chimiques pouvait être cause de la transformation de L'amidon en dextrine et glucose, et je me suis convainçue, par une foule d'expériences consignées dans mon Mémoire, que cette transformation etait uniquement efféctué par la saliva et je suis arrivé aussi à la découverte d'un principe actif analogue à la diastase par ses propriétés physiques et chimiques que je vais faire conaître. Le principe actife de la salive est solide, blanc, ou blanc-grisâtre amorphe, insoluble dans l'alcool, soluble dans l'eau et l'alcool faible. Ce principe est sans action sur les substances azotées, fibrine, albumine, caseine, gelatine et glutin * * * * * L'energie de ce principe est telle que une partie en poids suffit pour liquéfier et convertir en dextrine et en sucre plus de 2000 parties de fécule * * * * * il existe précisément un corps qui exerse sur l'amidon un pouvoir spécifique absolument semblabable à celui du ferment salivaire et ce corps est la diastase ou principe actife de l'orge germe decouvert par M. M. Payen et Persoz."

Mialhe also states that acids, alkalies, salts of copper, mercury, and silver destroy the fermentative power of saliva and malt diastase. He describes the precipitation of ptyalin by alcohol which he evaporated at 40° to 50° C. to obtain his "diastase salivaire" in a dry condition. He has also noticed that ptyalin in a dry condition may be kept for a long time without losing its diastatic power. He calls ptyalin "Diastate animale ou salivaire." Bouchardat[27] likewise examined the influence of alkalies, salts of copper, mercury silver, etc., upon the amylolytic action of saliva.

Magendie[28] has experimented with the parotid and with the mixed saliva of the horse and reports: "* * * * * les produits organiques sont une matière soluble dans l'alcool, une matière insoluble dans l'alcool et soluble dans l'eau * * * * * c'est la ptyaline ou matière salivaire * * * * * D'apres ces faits la salive n'est pas comme l'ont dit un grand nombre d'auteurs un liquide ne servant qu'a mouiller les aliments, et agissant simplement comme le ferait de l'eau distillée, en dissolvent les matièrs; mais elle joue un rôle chimiques dans le premier acte de la digestion."

Mialhe was, however, not the first one who had isolated ptyalin from saliva, but he was the first one to claim that the amylolytic action of saliva was due to the salivary diastase. The first investigator who isolated ptyalin was, it appears, Berzelius,[29] the celebrated chemist. He, like others, precipitated it by alcohol, but he evaporated at high temperatures and, therefore, could not recognize the amylolytic action of ptyalin as he destroyed it by the high temperature he used in evaporation. The proportion of ptyalin to the saliva in which it occurs is variously estimated by different observers. The great discrepancies in these different estimates are of course not at all surprising today.

The different estimates given are:

By Mialhe (l. c.)	2 per mille	Quoted from Hammersten Lehrbuch d. Physiol. Chemie, 1891, p. 143.
By Berzelius	3.8 per mille	
By Jacubowitsch	1.34 per mille	
By Frerichs	1.42 per mille	
By Herten	3.27 per mille	
By Hammerbacher	1.4 per mille	Poggend. Annalen Vol. 27, p. 320.
By Mitscherlich	1.10 per mille	
By Wright[30]	1.8 per mille	

The most extensive monograph upon saliva was written by Wright[30] and published through three successive years in the London Lancet

(1841 to 1844). This clinician examined saliva in health and in disease and he tried to utilize the results of his experiments for clinical purposes. He also injected considerable quantities of saliva (several drachms) into the jugular veins of dogs, producing thereby acute infectious diseases ending as a rule in the death of the animals so treated. Wright was under the impression that he had produced hydrophobia and he, therefore, ascribed to the human saliva properties of an eminently poisonous nature. We know to-day why human saliva injected into the blood current of animals is apt to produce acute infectious diseases. Of the many inferences which Wright draws from his extensive series of experiments with saliva few hold good today; but it seems that he was the first one who, still earlier than Mialhe, recognized clearly a number of influences damaging to the amylolytic action of the saliva. He states that acids or alkalies added to saliva diminish or destroy its digestive properties.

Concerning the special nature of the sugar formed by the action of saliva on starch, improper views were held for a long time.

For a time the sugar formed was accepted as glucose. Nasse[31] is responsible for a confusing dualism which has found its way into our views concerning ptyalin and malt diastase. This physiologist claimed that by the action of saliva on starch and glycogen a special kind of sugar was formed; he called this sugar amylum-ptyalose and glycogen-ptyalose respectively and he claimed that malt diastase and salivary diastase were two things absolutely different from each other. A number of years ago investigators who wrote after Nasse, proved that the claim of the latter could not be maintained because the product of salivary fermentation upon starch was maltose. Such writers were Musculus and Mering,[32] Brown and Herron[33] and Kuelz.[34] In spite of their publications a number of books still reproduce Nasse's mistake. This has induced Kuelz again to investigate the subject and his and Vogel's[35] extensive investigations upon the subject have been published. The two experimenters have caused saliva to act under the following varied conditions:

Parotid saliva acting on starch.
Mixed saliva acting on starch.
Pancreatic juice of the dog acting on starch.
Parotid saliva acting on liver-glycogen.
Parotid saliva acting on muscle-glycogen.
Pancreatic juice of cattle acting on liver-glycogen.
Pancreatic juice of cattle acting on muscle-glycogen.
Malt-extract acting on glycogen.

The products of these fermentations under varied conditions were isolated from their solutions in shape of their osazon by boiling with muriate of phenylhydrazin and acetate of sodium. The osazons so obtained were examined as to their solubility, as to their melting point, as to their crystallization macroscopically and microscopically, and were finally subjected to a quantitative analysis. The product always was found to be either maltosazon or a mixture of maltosazon and isomaltosazon. The product of the fermentation, therefore, was in each case maltose and isomaltose and in no way different from the product of the action of malt extract upon starch or glycogen. We may, therefore, adopt it as a fact that there is absolutely no generic difference between malt diastase and salivary diastase and all that remains in difference is a slight difference in their optimum temperature of development.

While the French investigators obtained ptyalin from saliva directly by alcoholic precipitation, Conheim[36] adopted a more complicated course —he precipitated with tribasic phosphoric acid and lime water. He found that slight quantities of acid in the starch pastes prevented the amylolytic action of ptyalin, which could, however, be reëstablished on neutralization. He found that the ferment was irredeemably destroyed only after considerable quantities of acids had been used. He also found that pepsin itself had no influence on ptyalin and that it was the free acid of natural or artificial gastric juice which damaged ptyalin and prevented its action.

A number of investigations about the optimum of the amylolytic action of ptyalin and diastase have been made by several observers. Kjeldahl (l. c.) found an optimum temperature of 46° C. for the amylolytic action of saliva. Bourquelot,[37] by a number of experiments, arrived at the conclusion that temperatures of 58° C. and higher damage saliva. He says: "* * * * * que si l'on admet qu'a partir de 58° C. environ la diastase salivaire commence à être détruite partiellement ou attenouée Cette attenuation va d'ailleurs en augumentant et vers 71° C. la diastase a perdu toute activité."

Bourquelot[38] also studied the action of higher temperatures upon diastase and he finds: "ainsi la diastase affaiblie même employé en excès a perdu le pouvoir dépousser la degradation de l'amidon j'usqua sa dernière limite. D'autre part, cette même diastase affaiblie accomplit les premières phases de la reaction aussi rapidement que la diastase naturelle * * * * on se trouve amené à penser que ce n'est pas la quantité du ferment qui diminue mais, que c'est sa qualité qui c'est modifié, à moins pourtant que l'on admette qu'il y a dans la diastase naturelle deux ou plusieurs ferments solubles mélangés et que l'élevation de temperature les detruit succesivement." Bourquelot found that diastase warmed for two hours at 68° C. did form very little sugar, but as much erythrodextrin as diastase which had not been heated to this degree.

Biernacki[39] has investigated the influence of higher temperatures on the digestive ferments. He states that fresh unfiltered saliva is destroyed at a temperature of 75° C. and filtered saliva at 70° C. He has, however, evidently taken into consideration only the formation of sugar and not noted that there may be a conversion into dextrin without any formation of sugar.

Schwarzer[40] in his experiments found that diastase at 65° C. suffers a diminution of amylolytic power, he also found that the higher the temperature above 65° C. and the longer it acts upon diastase the more is it weakened. He expresses his belief that the amylolytic power of malt extract may be destroyed by keeping the same for a sufficient length of time at 70° C. Dumas[41] states that borax neutralizes the action of diastase, but as he has made his experiments at a temperature of 70° C. they cannot be accepted as conclusive evidence because this temperature in itself destroys, if acting long enough, the amylolytic power of the diastatic ferment. Bassewitz[42] has ascertained that the amylolytic action of the diastatic ferment is increased in the presence of carbonic acid gas, but that atmospheric pressure has a hindering influence. Falk[43] has noticed that acids are inimical to the amylolytic ferments and that they are destroyed by the action of gastric juice but not by pepsin alone without HCl. Detmer,[44] Stutzer and Jubert,[45] Ellenberger and Hoffmeister[46] have all examined the influence of various agencies upon the amylolytic ferments, they have all noticed that acids are very hurtful, especially the mineral acids. The most careful studies of the modifications of the diastatic action of saliva under a variety of conditions such as addition of acid alkali, different neutral salts and other metallic salts, peptone, etc., were made by Chittenden[47-52] and his co-workers in a large series of experiments published in a number of articles. Chittenden and his co-workers in numerous experiments also established the fact that proteid matter (peptones) and various salts increase materially the action of amylolytic ferments upon starch. Langley and Eves[53] also found that peptones increase the amylolytic action of diastatic ferments. A very peculiar kind of diastase seems to flourish in St. Petersburg, provided that a report about Mollschkowsky's[54] inaugural dissertation found in the Higienische Rundschau does not misquote. According to his report Moltschkowsky found that an addition of corrosive sublimate in a proportion of 1: 200,000 totally destroys the amylolytic action of saliva. This is very far from being true; on the contrary such small additions of bichloride of mercury hasten and favor the fermentative action of saliva as the author's experiments reported below will show. Ptyalin in this respect behaves exactly like yeast, which according to Biernacki[55] also shows an increased activity in the presence of small quantities of bichloride of mercury. Only very few observers seem to have made experiments with the amylolytic ferment filtered through Pasteur filters. Krabbe[56] reports that malt diastase filtered through a bacteria filter has a very weak action. He also tried to filter through clay cells used for electric batteries, but only after the use of pressure could he find any diastatic action in the filtrate.

A number of chemists have tried to offer satisfactory chemical theories and formulæ explaining from a purely chemical standpoint the conversion of the starch molecule through a number of intermediary changes into the sugar molecule. Musculus[57] arrived at the conclusion "que la saccharification de cette substance sous l'influence de ces agents doit être considerée comme un phenomène de dédoublement, c'est à dire que l'amidon ne se transforme pas d'abord en dextrine par changement isomerique, puis en sucre par hydration come on admettait alors mais que la dextrine et le sucre se produisent simultanément, qu'il y a hydration suivie de dédoublement en dextrine et sucre." Musculus and Gruber[58] later worked out a series of changes they assume as taking place simultaneously and successively. They give the following conclusions:

"Voici maintenant la liste des differents corps qui se produisent aux dépens de l'amidon sous l'influence de la diastase ou de l'acide sulfurique dilué et bouillant.

1. Amidon soluble		pouvoir rotatoire $\alpha]_D$ =	+ 218°
		pouvoir réducteur =	6°
2. Erythrodextrine			
3. Achroodextrine			
	α	pouvoir rotatoire $\alpha]_D$ =	210°
		pouvoir réducteur =	12°
	do β	pouvoir rotatoire $\alpha]_D$ =	190°
		pouvoir réducteur =	12°
	do γ	pouvoir rotatoire $\alpha]_D$ =	150°
		pouvoir réducteur =	28°
4. Maltose: Formula $C_{12} H_{22} O_{11} + H_2O$			
		pouvoir rotatoire	150°
		pouvoir réducteur	66°
5. Glucose: Formula $C_6 H_{12} O_6 H_2O$			
		pouvoir rotatoire	57°
		pouvoir réducteur	100°

Nous considerons donc l'amidon comme un polysaccharide de la formule

$$n\ (C_{12} H_{20} O_{10})$$

dans lequel le valeur exacte de "n," qui ne parait pas être moindre que 5 ou 6, reste a déterminer.

Sous l'influence des ferments diastasiques et des acides dilués cet hydrate de carbon, subit une serie d'hydratations et de dédoublements succésifs. À chaque dédoublement il se forme de la maltose, et une nouvelle dextrine, à poids moleculaire plus faible; "n" devient de plus en plus petit jusqu'a production de l'achroödextrine "γ" laquelle se transforme probablement en maltose par simple hydratation. La maltose s'hydrate ensuite à son tour et se dédouble en deux molécules de glucose suivant l'equation

$$C_{12} H_{22} O_{11} H_2O = 2 (C_6 H_{12} O_6)$$

Brown and Herron[59] independently of Musculus and Gruber and about the same time arrived at similar conclusions, though the optical and other properties of their different dextrins do not at all coincide with those found by Musculus and Gruber.

The different dextrins given by Brown and Herron are:

Soluble starch $[\alpha]_D$ =	216.0°
Erythrodextrin α	209.0°
do β	202.0°
Achroodextrin α	195.4°
do β	188.7°
do γ	182.1°
do δ	175.6°
do ε	169.0°
do ζ	162.6°
do η	156.3°
Maltose	150.0°

The conclusions at which Brown and Herron arrive concerning the molecular structure of starch is that the molecule is represented by the formula

$$10 (C_{12} H_{20} O_{10})$$

The first effect of the ferment upon this molecule according to Herron and Brown, is that one of the ten groups $(C_{12} H_{20} O_{10})$ is withdrawn from the original molecule by hydration and formation of maltose. The remaining nine groups of $(C_{12} H_{20} O_{10})$ form the first dextrin of the series; i. e., erythrodextrin α. This molecule again loses one of its nine groups by hydration; i. e., formation of maltose, and a molecule of the eighth group representing erythrodextrin β remains. This process is repeated and repeated again until nothing but the last dextrin of the series, representing only one group of $(C_{12} H_{20} O_{10})$, is left.

According to Maerker[60] four starch molecules at 60° C. give three molecules of maltose and one of dextrin; at 65° C. the quantity of maltose is somewhat reduced (?) and at still higher temperatures two molecules of starch furnish one molecule of maltose and one of dextrin. Maerker is inclined to believe that what is called malt diastase contains two different enzymes of which one produces a great deal of maltose and a small amount of dextrin and is destroyed at comparatively low temperatures and the other enzyme a good deal of dextrin and a small amount of maltose and is only destroyed at a higher temperature. The second enzyme does not act at all upon dextrin but only upon starch.

O'Sullivan (i. c.) has laid down three equations as referred to above, they are:

At temperatures below 63° C.

$$\text{A.}\quad C_{18}\,H_{30}\,O_{15} + H_2O = \underset{\text{Maltose}}{C_{12}\,H_{22}\,O_{11}} \qquad \underset{\text{Dextrin}}{C_6\,H_{10}\,O_5}$$

At temperatures between 64° and 68° to 70°.

$$\text{B.}\quad \left.\begin{array}{l} C_{18}\,H_{30}\,O_{15} + H_2O \\ \qquad\qquad\qquad = \\ C_{18}\,H_{30}\,O_{15} \end{array}\right\} C_{12}\,H_{22}\,O_{11} + 4\,(C_6\,H_{10}\,O_5)$$

At temperatures between 68° to 70°.

$$\text{C.}\quad \left.\begin{array}{l} C_{18}\,H_{30}\,O_{15} \\ C_{18}\,H_{30}\,O_{15} \\ C_{18}\,H_{30}\,O_{15} \\ C_{18}\,H_{30}\,O_{13} \end{array}\right\} + H_2O = C_{12}\,H_{22}\,O_{11} + 10\,(C_6\,H_{10}\,O_5)$$

A number of chemists have tried to analyze the body known as malt diastase. Most of those who have tried it have confessed that they were unable to obtain diastase in a condition sufficiently pure to permit of an exact quantitative analysis. Two analyses are, however, given in literature. They give the following figures:

Analysis of Zulkowski and Renner[61]		Analysis found in Watt's Dictionary[62]	
C.	47.57	C.	44.33
H.	6.49	H.	6.98
N.	3.16	N.	8.92
O.	37.64	O.	32.91
Ash	3.16	Ash	4.79
S.	a little	S.	1.07
	98.02		99.0

These figures do not at all agree with each other.

In concluding this historic review of the literature on saliva and on the amylolytic ferments of malt and salivary diastase, I cannot refrain from citing one article which appeared in Virchow's Archives (S. de Jager[63]), an article which demonstrates how unsatisfactory has heretofore been our knowledge concerning the unorganized ferments. In this article the writer in all seriousness tries to prove that enzymes are not bodies at all but that they belong to the class of "imponderabilia" to which belong light, heat, electricity, magnetism. The property of the enzymes to lose their fermentative power on being heated he likens to the property of the magnet to lose its magnetism when exposed to higher temperatures.

In the following experiments the main object was to demonstrate that the enzymes malt diastase and salivary-ptyalin are not as has heretofore been believed, dead, unorganized bodies, but elementary organisms, probably very small ultramicroscopic granules which produce in their process of growth and multiplication as the product of their metabolism upon starch dextrins and sugar. In the quantitative estimation of the amylolytic action the sugar found was always calculated as maltose, of which 154 mgr. will reduce all the copper hydrate contained in 20 c. c. of Fehling's normal solution.

In the conversion of starch by diastase and ptyalin the end product probably, however, contains not only maltose and isomaltose but also some dextrose or glucose, which has a very much higher reducing power than maltose. Besides, the different dextrins formed, may possess a certain amount of reducing power. Concerning the latter point authors differ very much in their statements. Be this as it may, in the following experiments the amount of reducing substances formed has always been calculated as maltose, of which as stated before, 154 milligrams will reduce all the copper hydrate in 20 c. c. of Fehling's normal solution. The figures obtained, which have, for convenience sake, been always designated as "amount of sugar formed," are all too high, because in the first place, there was also probably some dextrose of a very much higher reducing power formed, and the dextrins formed, having probably also some reducing power, are necessarily included in the estimate. For these reasons all the figures given are too high. This, of course, does not disturb their mutual relations to each other. It is claimed that diastase and ptyalin can never convert all the sugar in a solution up to the end product of fermentation; namely, sugar, and that always a certain percentage of dextrins remain unchanged, no matter how small the percentage of starch in the solution, nor how large the amount of the ferment added. There is no doubt, of course, that we can in solutions not too concentrated, convert all the starch; i. e., make all of the starch disappear and change it into dextrins and sugar; but all the evidence collected tends to show that a complete conversion of all the starch into sugar is impossible.

It has been known for a number of years that there are microorganisms which possess an amylolytic action, which has been ascribed to the formation of the diastatic ferment by these organisms. It appears that Wortmann[64] was the first who made more extensive observations upon this point. Vignal,[65] who has made a special study of the microorganisms of the mouth, is under the impression that one of these organisms possesses a slight amylolytic action. He says, concerning this point: "J'ai cherché l'action qu'exèrcent sur l'amidon un certain nombre de microörganismes que j'aie isolées l'anée dernière de la bouche et les deux que j'aie trouvée depuis.

Parmi ces microorganismes * * * * * trois transforment l'amidon. mais un seul agit un peut énergiquement, un autre parâit vivre à ses dépens sans l'hydrate."

From this notice it remains doubtful whether there really is present in the mouth a microorganism with an undoubted amylolytic action.

Still in experiments the purpose of which it is to show that the enzymes diastase and ptyalin are elementary organisms or ultramicroscopic granules multiplying in the proper media and producing as the result of their growth in a nutrient soil containing starch dextrins and sugar it is necessary to exclude the possibility of this action being brought about by microorganisms such as bacteria, yeast-cells, moulds with an amylolytic action. The method employed to obtain a malt extract and a saliva free from any microorganisms will be described below.

ANALOGIES BETWEEN MICROORGANISMS AND ENZYMES

If one compares a number of general observations made about enzymes with a number of general observations made about microorganisms one cannot help being very much impressed with the many points of analogy which have been clearly established, but to which nobody up to the present time has called attention. Such points of analogy are:

1. The propagation of microorganisms (bacteria, schizomecetes, saccharomycetes) depends upon certain temperatures and occurs only within the range of certain limits of temperature. The activity of the enzymes likewise depends upon certain temperatures ranging over the same territory as those ascertained for microorganism.

2. All bacteria and their spores if exposed to streaming steam of 100° C. are killed (many sooner); likewise all enzymes lose their fermentative power if so treated.

3. All microorganisms have optimum temperatures of growth development and propagation, all ferments act best at certain optimum temperatures.

4. Most microorganisms are killed by moist heat at temperatures lower than 100° C. The enzymes also lose their converting power at temperatures lower than 100° C. moist heat.

5. Microorganisms resist dry heat better than moist heat; likewise do so-called unorganized ferments.

6. Bacteria and other microorganisms will retain their power of propagation when in a dried state for a very long time. The same holds good for the fermentative action of enzymes. A watery diastase preparation will lose its amylolytic power very soon; dry diastase can be kept for years and retain its specific power.

7. The development of different kinds of microorganisms depends upon the neutral or acid or alkaline reaction, and its degree, of the culture medium. Different microorganisms have their varying limits of permissible alkalinity or acidity. The same rule holds good for the enzymes.

8. So-called antiseptics and all kinds of salts influence the growth of bacteria and prevent it if present beyond certain limits. The same holds good with reference to the special ferment action of the enzymes.

9. Agents of the above description inimical to the development of microorganisms if present in a larger proportion, often act as stimuli of development if present in a very minute proportion. The same is true with reference to enzymes. Chittenden and his co-workers have demonstrated that this is the case with reference to a number of salts inimical to the action of the amylolytic ferments in certain proportions and increasing the action in other smaller proportions.

10. The development of microorganisms in any soil can only be carried on to a certain extent (no matter how rich the soil) because the accumulation of the metabolic products puts a stop to further development as soon as those metabolic products are present in a certain percentage. The action of enzymes is always limited by the accumulation of the products of their fermentative action.

11. I have ascertained that the amylolytic ferments like many microorganisms are destroyed by repeated alternating freezings and thawings.

EXPERIMENTS TO DEMONSTRATE THE TRUE NATURE OF THE DIASTATIC ENZYMES

The following experiments were made to demonstrate that malt diastase consists of exceedingly small live elementary granules or organisms multiplying in starch paste and producing in the presence of starch as the product of their metabolism dextrins and sugar: Ground brewery malt was digested in a beaker with sterile distilled water in a water bath at a temperature of 45° C. for half an hour, the temperature was then slowly raised to about 60° C. and kept there until all the starch had been converted. This point, of course, can be ascertained by repeated tests with an iodine solution in iodide of potash. When all starch has been converted the mash will not be stained any more by iodine, neither blue, violet, nor red—achroodextrin and sugar giving no color reaction with iodine. After the starch had all been converted the mash was filtered through a paper filter into another sterile vessel and the filtrate, now an almost transparent yellowish tinted fluid, again heated for some time in a water bath at a temperature of 63° C. This temperature does not damage the diastatic ferment of the extract of malt but it destroys the peptonizing ferment, the peptase also contained in malt extract. If one makes a cover-glass preparation of a malt extract, prepared according to the above directions, just as one would make it in examining a bouillon containing microorganisms, and stains it with a saturated alcoholic solution of eosin one sees an enormous number of exceedingly small round granules.

Another method of staining the ferment granules, which furnishes excellent results, is that by the use of a "Beize," recommended by Bunge.[66] This cover-glass after having been drawn three times through the Bunsen flame is first treated with Bunge's "Beize," a fluid somewhat similar to Loeffler's mordant. It is then stained with Ziehl's carbol-fuchsin. This method is described fully in part 1.

When stained according to this manner the ferment granules show up beautifully, and it appears that they have a gelatinous capsule.

If a fresh so-called diastase solution be prepared and stained according to one of the above methods the small ferment granules are seen in large numbers. One also sees here and there grains of achroodextrin studded densely with these small bodies. If one now examines the diastase solution in the hanging drop one will in vain search for the granules. This is undoubtedly one of the reasons why they have not been seen before, and why it has also been claimed that diastase is a body perfectly soluble in water. If dry diastase is mixed with water, shaken, and filtered a clear product with diastatic power is obtained, hence the impression that something has gone in solution and that no corpuscular, cellular elements are present. The reason for the failure to see any cells in a hanging drop of water must be ascribed to the fact that diastase cells have the same or almost the same optical refraction index as water. Hence, they can, of course, not be seen in such a medium and must remain invisible. There are, however, means to be described presently, to make visible the cells in the hanging drop. For this purpose, however, a filtrate obtained after passage through a Pasteur filter is best used.

If a freshly prepared diastase solution is forced through the pores of a good, reliable Pasteur filter, an absolutely transparent, clear filtrate is obtained. Cover-glass preparations from such a filtrate show the small round granules and nothing else. Starch granules, bacteria, etc., are kept back by the filter, and only the very minute ferment granules pass its pores. These ferment granules can even pass the stomata of an animal membrane, as will be seen later on.

In a filtrate which has passed the Pasteur filter, and which is free from all other cellular elements but the ferment granules, the latter may be made to appear in the hanging drop in the following manner: To the filtrate is added, drop by drop, until a milky turbidity appears, either a saturated solution of picric or a weak solution of tannic acid. If a drop of this filtrate so treated is now examined under the microscope the small granules may be seen. They have probably become visible by a precipitate having formed on their surface. This precipitate is redissolved on heating and reappears on cooling. After the microscopic examination of a freshly prepared malt extract, a so-called dry diastase, prepared by Merck, was likewise examined in the same manner. It may be sufficient to state that this preparation, obtained by precipitating diastase by alcohol from a fresh watery solution, consisted of nothing else but the small cells mixed with grains of achroodextrin. In order to make cover-glass preparations from a dry diastase it is necessary to first mix with distilled water and filter, otherwise one will not be able to see any individual granules, because in the dry diastase they are agglutinated to each other in larger or smaller lumps. The reaction with picric acid and tannic acid is the same as it is for the freshly prepared diastase.

Here the objection may be raised that the reaction of the picric and tannic acids is nothing more nor less than the well-known reaction of these chemicals upon soluble albuminoid substances. It cannot be and it is not the object of this paper to go into the question of what every soluble albuminoid body is, but it is the object of this paper to show that the two so-called soluble albuminoid substances, the enzymes, malt diastate and saliva ptyalin, are not amorphous, unorganized substances, but are masses of living granules. To demonstrate this it was necessary to prove that these granules can be propagated as we can propagate bacteria, yeast cells, etc. The microscopic study alone could, of course, not furnish any conclusive evidence.

A circumstance which facilitates materially the following up and study of the growth and the multiplication of diastase and ptyalin granules is the fact that we are so well familiar with the products of diastatic enzymes.

The amount of sugar formed, the formation of one of the dextrins as ascertained by the iodine test, gives us reliable means to gauge with chemical exactness the progress in the metabolism and multiplication of the diastase and ptyalin ferment granules.

For the purpose of propagating these diastase granules a thick corn-starch paste was made, sterilized in flasks and test-tubes, and examined for sugar. After it had been ascertained that no appreciable amount of sugar was present, microscopic stained preparations were made from the starch paste.

EXPERIMENT I

A solid starch paste, contained in a large flask, was inoculated with a platinum needle from a freshly prepared malt extract on the periphery, and then kept in a water bath for half an hour at a temperature of 60° C. The surface of the paste was liquefied. The microscopic preparations made from the center of the surface showed enormous numbers of small round granules. The presence of an appreciable amount of sugar was proven.

EXPERIMENT II

In each of ten Pasteur flasks were placed one gram of cerealine and nine ccm. of distilled water. The flasks so prepared were then sterilized for two hours in steam of 100° C. A fresh diastase was then prepared under all aseptic precautions, and flask No. 1 was inoculated with a platinum needle from it. To ascertain how much material would be inoculated each time the platinum needle was weighed dry and dipped in a starch solution:

Weight of the platinum needle dry..................7.1425 grams
Weight of the platinum needle dipped in st. sol......7.1440 grams

The mass to be inoculated at one inoculation, therefore, weighed 1.5 milligrams. In order to avoid failure through loss of the little drop to be added, the inoculation was in each instance made three times and the platinum needle was sterilized over the Bunsen burner each time.

After flask No. 1 had been inoculated three times from the fresh malt extract its contents were well shaken and the flask was then transferred into the incubator and there left for an hour. It was then taken out and again well shaken and then No. 2 was inoculated three times from No. 1. No. 2 was then well shaken, left in the incubator for one hour, taken out, well shaken. No. 3 was next inoculated from No. 2, and so on, until No. 10 had been inoculated from No. 9. All the ten flasks were then left in the incubator for about two weeks. Their contents were then examined microscopically and chemically by iodine and Fehling's solution. Result: The small round granules in every flask in great numbers; starch liquefied; also sugar in every flask. The remainder of the contents of flask No. 10 was examined by titration for sugar and one-quarter of a gram of sugar was found. Each of the ten flasks contained one gram of starch, and to this amount in the first flask there was, in round numbers, about 1/100 ccm. of fresh malt extract added by inoculation with the platinum needle (3 times 1.5 milligrams = 4.5 milligrams, and in order to facilitate the calculation we will assume that 10 milligrams = 1 centigram was added). 1/100 gram in a flask containing 10 grams is equal to 1/1000 part of its contents. We, therefore, have in the first flask a proportion of diastase to starch equal to

$$1:100$$

We take the 1/1000 part of flask No. 1 and add it to No. 2; we, therefore, have in No. 2 a proportion of diastase to starch equal to

$$1:100 \times 1000$$

and in flask No. 3 we get a proportion of diastase to starch (of course always assuming now that an increase of diastase had not taken place, as has been taught heretofore.)

$$1:100 \times 1000 \times 1000 = 1:100 \times 1000^2$$

We find for every flask of the series the following proportions:

Flask No. 1	No. 2	No. 3	No. 10
1:100	1:100x1000	$1:100\text{x}1000^2$	$1:100\text{x}1000^9$

If we calculate the proportion of diastase to starch in flask No. 10, assuming that no increase had taken place from flask to flask, and if we assume that half of the starch in No. 10 had actually been converted into sugar, we find a converting power of

1:500,000 Billion Billions or one to 500,000 quadrillions.

The inoculation experiments from flask to flask may be a failure if one has not been very careful in preparing the malt extract and if one has not observed all the rules of the sterilization "technique." Then foreign microorganisms may develop, the starch paste may become sour, etc. These sources of disturbance may be avoided if instead of using a simply prepared malt extract a malt extract filtered through a reliable Pasteur filter be used for making the first inoculation. Such a filtrate prepared carefully does not contain any bacteria, yeast-cells, or moulds, as may be shown by pouring a number of plates from it. More about this subject will be said later, under the head of ptyalin.

EXPERIMENT III

The above experiments, No. I and No. II, were the first experiments made to demonstrate the organized live nature of the enzyme diastase, in them a very important point did not receive the proper attention, namely, the necessity to show that the diastatic action transferred from flask to flask was not dependent upon microorganisms possessing diastatic action. In the following experiments this point has always received proper attention, it has in fact been made the principal object of all control experiments. A large number of experiments carried on during a period of over seven months have taught me that the formation of maltose does not only act as a hindering agent to the further development of the diastase granules but that it will probably even exert a destructive influence if acting for a considerable time. We know that many microorganisms will be destroyed in a comparatively short time by their own metabolic products, so that a culture medium in which a microorganism of a special description has been developed will after the lapse of a certain time become sterile and a propagation of the special microorganism from it will be an impossibility. The most striking example of this kind is perhaps furnished by the diplococcus pneumoniæ of Fraenkel. It is well known how rapidly this microorganism dies out in a culture and at what short intervals of time it is necessary to transfer to fresh soils lest the power of propagation will be lost. It seems evident to me from a long series of experiments that maltose exerts a certain destructive influence over the diastase granules if acting upon them for weeks and months.

Diastase granules do not multiply only in a soil containing starch but they multiply even very much better in an albuminoid soil free from starch. The first experiments by which this fact was demonstrated were undertaken with the salivary ptyalin granules and they will be reported further below.

The observation that the diastatic ferment granules do multiply very rapidly in an albuminoid nutritive soil free from starch has, in connection with a method to be described presently, furnished an excellent arrangement to propagate from generation to generation.

This method to obtain pure cultures is the dilution method first employed by Pasteur to obtain pure cultures of yeast cells and improved by Emil Christian Hansen. It must be stated, however, that with reference to the diastatic ferment granules I could avail myself only of the general principle of the dilution methods, because these granules do not form any colonies and they are so small that any attempt to start from a single granule by a method of counting them in a quantity of fluid would be absolutely futile. The experiment based upon the observation that the diastatic ferment granules multiply in an albuminoid medium and upon the dilution method was made in the following manner.

A sterile flask containing several grams of dry ground brewery malt was kept in a water bath at a temperature of 63° C.; another flask containing about 100 c. c. of sterile water to which had been added 10 c. c. of a peptone-glycerine-bouillon (as used in bacteriological work) was likewise kept under the same conditions. When both flasks were long enough in the water bath to justify the expectation that both their contents were brought up to about 63° C. the water-bouillon mixture was poured into the flask containing the dry ground malt. This flask was now left in the water bath for one hour, always keeping the temperature of the bath at or near 63° C. After the lapse of an hour the malt extract so prepared was filtered through a sterile paper filter into another sterile flask and as soon as a few c. c. filtered malt extract had been collected, the flask containing the same was again transferred to the water bath and kept there for half an hour at 63° C. This method of preparing the malt extract was dictated by assuming that at a temperature of 63° C. very few microorganisms would develop, while the development of diastase, of course, is at its optimum at 63° C.

That this assumption is correct may be proven not only by making stained microscopical preparations from such a malt extract, but better still by pouring plates from it. In the experiment described, the plates poured from this malt extract developed on an average 1-2 colonies on each plate and a few plates even did not develop any colonies at all. If microscopical stained specimens be examined, occasionally a stray microorganism, but an enormous number of the small round ferment granules will be seen. After the malt extract had been filtered and the flask containing the filtrate had been replaced in the water bath, a number of test-tubes containing 10 c. c. of a glycerine-peptone-bouillon were likewise placed in the water bath. The bouillon was prepared according to the ordinary methods used in bacteriology only that the salt used to neutralize was not bicarbonate of potash but diphosphate of sodium.

After the flask containing the filtrate had been kept for ½ hour in the water bath, from it an inoculation with a platinum needle was made to test-tube No. 1; after ten minutes No. 2 was inoculated from No. 1 and so forth until No. 10 had been inoculated from No. 9. All ten test-tubes were then placed in an incubator and kept there at a temperature of about 50° C. for seven days. After the lapse of this time the contents of each of the ten tubes were poured over to flasks containing 90 c. c. of a starch paste (2 grams starch: 90 c. c. water). It is hardly necessary to state again that all manipulations were undertaken with the strictest observance of all the rules of the sterilization technique.

After the lapse of five days plates were prepared and inoculated from all of the ten flasks; these plates were kept in the incubator and examined daily. The result of this bacteriological control experiment may be stated here:

The plates inoculated from flasks No. 1, No. 2, and No. 3 developed numerous colonies. The plates from flask No. 4 developed very few colonies. The plates inoculated from flasks No. 5 to No. 10 developed no colonies of any kind. (The soil used for the plates which were poured into Petri's dishes was glycerine-agar.)

After plates had been inoculated from the contents of all of the ten flasks they were taken from the incubator and sterilized in the steam sterilizer in order to arrest any further ferment action. The contents of the flasks were now examined by the iodine test and by titration with Fehling's solution for sugar. The result of this examination was the following:

No.	Iodine test	Reaction	Amt. of sugar	Micro-organisms
1.	blue	slightly acid	0.075	many
2.	blue	slightly acid	0.071	many
3.	blue	slightly acid	0.077	many
4.	violet-blue	slightly alkaline	0.933	few
5.	violet-portwine red	slightly alkaline	1.519	none
6.	violet-portwine red	slightly alkaline	0.802	none
7.	violet-portwine red	slightly alkaline	1.540	none
8.	violet-portwine red	slightly alkaline	1.510	none
9.	violet-portwine red	slightly alkaline	1.026	none
10.	blue	slightly alkaline	none	none

This table shows the following: In diluting, at test-tube No. 5 a stage had been reached when no more microorganisms were present but ferment granules were present. In No. 10 there were neither microorganisms nor ferment granules present. The microorganisms found in the first flasks could not have been the carriers of an amylolytic action, they on the contrary evidently prevented, if present in large numbers, the action of the amylolytic ferment.

EXPERIMENT IIIB

A number of experiments were made to ascertain the power of resistance of the diastase granules to high and low temperatures, etc. Against alcohol, diastase is very resistant. This was to be expected because dry diastase is prepared from the fresh malt extract by precipitation by alcohol. A few grams of dry diastase (Merck's preparation) were immersed in strong alcohol (93%) and left in it for twenty-four hours, being frequently shaken while so immersed. After the lapse of this time the alcohol was poured off, the diastase dried and added to starch paste, which was after the lapse of some time completely converted into sugar and achroodextrin.

Malt extract and saliva were then, at a time when the temperature ranged from minus 5° to minus 15° C. kept in the open air during twenty-four hours. During this time the frozen fluids were three times taken in and thawed. The amylolytic ferments so treated were all absolutely dead; they could not in thirteen days bring about any change upon starch pastes kept in the incubator.

SALIVA AND SALIVARY DIASTASE (PTYALIN)

The saliva used in the following experiments was mixed saliva derived from my own buccal cavity. The latter was first cleansed by brushing the teeth and rinsing the mouth with dilute alcohol, a weak sublimate solution, and finally sterile water. The flow of saliva was stimulated by smelling lemons, sulphuric ether, and glacial acetic acid, the latter always brought about the most abundant flow.

I want to state right here a few general observations made in the course of my experiments, observations the knowledge of which is necessary in order to avoid sources of error in making comparative tests:

Saliva possesses different degrees of amylolytic power at different times.

A difference of amylolytic power is noted in case of a small quantity of saliva collected and in case of a larger quantity, being obtained by all kinds of stimuli. The saliva secreted and collected when left standing at a moderate temperature first increases for a number of hours in amylolytic power, this power then diminishes rapidly finally to disappear completely.

It is, therefore, necessary in making comparative experiments always to make use of the saliva of a single crop and to hasten a given series of experiments as much as possible. It would not do in ascertaining the optimum temperature of the amylolytic activity to work with a number of temperatures today and another batch of temperatures tomorrow, nor would it do to use different quantities of saliva collected at different times.

As saliva is more or less viscid it would be almost impossible to measure off one or two cubic centimeters in an exact manner. In the following experiments the saliva used was, therefore, collected in a sterile flask and then well mixed by energetic continued shaking with four or nine times its volume of distilled sterile water. In this manner a twenty or a ten per cent mixture of saliva was obtained; of this 5 or 10 ccm. were added to the starch solutions. All experiments were made under the strictest observance of the rules of the sterilization technique.

The study of the ferment granules is made difficult by circumstances referred to above, but the study of ptyalin and malt diastase ferment granules is very much facilitated because we know so well the products of their fermentative action, or in other words, the product of their metabolism. The dextrins and the sugar formed which we can measure quantitatively by Fehling's test or by splitting the sugar up into alcohol and CO_2, give us an exact estimate of the multiplication of the ferment granules in question. These chemical tests have, therefore, been made in the following experiments as carefully as possible.

In estimating the amount of sugar formed by titration, the point when all the cupric hydrate in Fehling's solution had been reduced was not only ascertained by observing when the test fluid became clear, but also by the additional testimony of the cessation of the formation of ferro-cyanide of copper. (Acetic acid-ferro-cyanide of potash test.) In some instances the amount of sugar ascertained by titration was further controlled by the fermentation test; i. e., to the mash containing sugar, yeast cells were added and the sugar so split up into alcohol and CO_2. From the amount of CO_2 formed and the consequent loss in weight, the amount of sugar previously held in solution may be easily calculated.

Saliva as it is obtained from the buccal cavity is not very well adapted for a microscopic examination with reference to the ptyalin granules. Such saliva contains too many different cellular elements (epithelial cells, microorganism, etc.). But excellent preparations may be made from a filtrate which has passed the Pasteur filter (of course everything always to be made under aseptic precautions, sterilization of the filter, collecting the filtrate in a sterile flask, etc.) and from a dialysate made in a manner to be described presently.

A piece of small gut of the lamb such as is used for making sausages is first kept for a number of hours or days in a corrosive sublimate solution. It is then drawn over the upper end of a test-tube and securely fastened to it by means of surgical silk. The lower end of the test-tube is to be opened by cutting the bottom out. This opening is then closed by a plug of cotton and the dialyser so prepared is as a whole sterilized in the steam sterilizer. After being sterilized a few c. c. of saliva or of malt diastase which have been cooled in the ice chest are filled into the test-tube. This is at once immersed in a flask containing sterile water to which a few c. c. of bouillon have been added. The dilute bouillon is to be warmed to about 50° C. before the test-tube dialyser is immersed in it. The difference in the temperature of the fluids in the test-tube dialyser and in the flask and the difference in the specific gravity of both quickly brings about an osmotic interchange and with it a migration through the stomata of the membrane of the diastatic granules. I have had the test-tube dialyser immersed from 1 minute to 2 days but always could show a diastatic action of the dialysate. It requires a good deal of nicety and a very short time of immersion to get a dialysate which is free from microorganisms. But this can be accomplished by this method because microorganisms cannot, if the membrane has been properly and tightly fastened, escape out through the membrane. The latter is, of course, if kept long enough in the flask, finally destroyed by microorganisms.

While a dialysate is not always free from microorganisms and so cannot be utilized to propagate from it in pure culture, it will always make a good material for microscopic preparations because it shows the ferment granules in sufficiently small numbers to be well observable individually. A number of preliminary experiments were made to show that ptyalin granules can be propagated from generation to generation, but as in all those experiments the amount transferred from flask to flask was always 1 c. c. these experiments will not be reported in detail as they might give rise to objections.

Experiment IV

This experiment was made with a filtrate of saliva obtained by filtering it through a Pasteur filter. Plates poured from this filtrate did not develop any bacteria, saccharomyces, or mould colonies. In filtering saliva through a Pasteur filter it is necessary to first dilute it with four to nine times its volume of distilled sterile water, otherwise it takes too long until one gets a filtrate.

The inoculations in this experiment were again made with a platinum needle. The flasks to be inoculated contained each 2 grams of starch to 100 c. c. water. No. 1 after having been inoculated was left in the incubator for twenty-four hours, then No. 2 was inoculated from No. 1, etc., until No. 5 had been inoculated from No. 4. Five days after No. 5

had been inoculated from No. 4 and when the contents of every flask showed signs of an advanced liquefaction of the starch, plates were poured from each flask and kept in the incubator at temperatures of 25° C. and 40° C. These plates did not develop any bacteria, saccharomyces, or mould colonies.

The contents of the five flasks were examined by the iodine-iodide of potash solution for dextrins, and by Fehling's solution (titration) for sugar. The test for dextrins is made in the following manner: From a weak iodine solution in iodide of potash (of a deep yellow to a light yellow-brown color) drop by drop is added to some of the fluid to be tested; if this fluid contains the different dextrins their respective colors appear successively. Amylodextrin gives, like starch, a blue; erythrodextrin, a port wine coloration; while achroodextrin shows only the original yellow of the iodine solution. If a solution contains all the three dextrins and the iodine test fluid is added drop by drop, the play of color appears in the following order: blue, blue-violet, violet, deep violet, port wine brown. In this manner the presence of the different dextrins may be ascertained. All the five flasks in the above experiments contained the dextrins. The following amounts of sugar were ascertained for each one of the flasks:

No. 1	0.700 grams
No. 2	0.171 grams
No. 3	0.220 grams
No. 4	0.128 grams
No. 5	0.096 grams

After the presence of dextrins and sugar had been ascertained, the remaining contents of each flask were brought to boiling over a Bunsen burner in order to arrest any further action of the ptyalin cells. 50 ccm. of each flask were then poured into a fermentation bottle and some yeast added. A fermentation made itself manifest and the gas formed was conducted through a rubber tube into a flask containing a solution of caustic barium. Carbonate of barium was precipitated proving that the gas formed was carbonic acid. The reducing substance contained in the five flasks of this experiment was, therefore, identified as a fermentable sugar.

I may here insert that in spite of every precaution it may happen that accidental microorganisms may develop in the starch-paste, while it is being converted into dextrins and sugar by the amylolytic ferment granules, even when a Pasteur filtrate is used. If such is the case, if colonies show on the plates inoculated from the contents of the fermenting starch-paste, it is necessary to prove that these microorganisms appearing incidentally are not the carriers of an amylolytic fermentative power. Sterile starch-paste must then be inoculated carefully from the top of such colonies. In doing so one must be particularly careful to inoculate from the top of a colony without touching with the platinum needle the surface of the solid culture soil upon which the colony is growing. Why this precaution is necessary will become evident by some experiments to be reported presently. A short movable bacillus found in the saliva grows frequently in starch solutions, but this bacillus has no diastatic action, it produces on the contrary an acid, and, therefore, is apt to arrest amylolytic changes.

The question now arises whether the ptyalin granules grow and multiply in an albuminous soil free from starch. Plates were poured (inoculated from a Pasteur filtrate of saliva) and were left in the incubator for a considerable length of time (up to two weeks). They did not develop colonies of any kind. If starch-paste was added to such plates poured into flasks, after twenty-four hours there was a considerable amount of sugar formed, much more than if the starch solution was added to a plate which had been inoculated immediately before the starch solution was added.

The following

Experiments V and VI

will illustrate the propagation of the ptyalin cells in albuminous soils free from starch. A tube containing glycerine agar was inoculated from a Pasteur filtrate of saliva and a plate was poured from it into a flask. This plate was left in the incubator for four days (40° C.). After four days 100 ccm. of a 2% starch solution was added to it and after two more days the amount of sugar was ascertained to be 0.164 grams. When to the plate, four days old, the starch solution was added, two test-tubes with glycerine agar were inoculated from a filtrate and to each was at once added 100 ccm. of a 2% starch solution. The titration for sugar was made after two days. The amounts ascertained were:

0.051 grams and 0.065 grams.

The second experiment to prove the growth and multiplication of the ptyalin granules in an albuminous nutritive soil was the following: To 100 ccm. distilled sterile water and to 100 ccm. of a glycerine-peptone-bouillon was added one ccm. of fresh saliva. Both bottles were then left in the incubator for eight hours (40° C.). Then one ccm. was taken from each of the two and added to 100 ccm. of a 2% starch solution. The starch-paste so inoculated was left in the incubator and after ten hours the amount of sugar formed was ascertained:

Amount of sugar in the 100 ccm. starch solution inoculated from the glycerine-peptone-bouillon, 0.110 grams.

Amount of sugar in the 100 ccm. starch solution inoculated from the distilled water, 0.010 grams.

After thirty-three hours two bottles each containing 100 ccm. of a 2% starch solution were again inoculated in the same manner and then left in the incubator for ten hours.

Amount of sugar in the bottle inoculated from the glycerine-peptone-bouillon, 0.575 grams.

Amount of sugar in the bottle inoculated from the distilled water, traces.

After fifty-five hours the experiment was repeated again, but now both starch solutions inoculated yielded only traces of sugar. Evidently the ptyalin granules which had grown and multiplied in the peptone-bouillon had been destroyed. There had, of course, grown in this soil microorganisms which had been imported with the saliva (no filtrate, but an unfiltered saliva having been used). These microorganisms growing in great numbers in the fluid culture medium had, therefore, evidently destroyed in some way the ptyalin granules that were developing. The ptyalin granules do not multiply on a solid glycerine-agar soil in the form of colonies; it seems that they form a very fine veil but this cannot be resolved into single colonies by the aid of the microscope. The ptyalin granules do not, of course, liquefy gelatine-peptones as they have no peptonizing power.

Propagation experiments with an aseptic dialysate were made in the following manner:

A number of flasks partly filled with glycerine-peptone bouillon into which were projected test-tube dialysers in such a manner that the membrane closing the tube would be still above the level of the bouillon—were sterilized for two hours in the steam-sterilizer. After the gas had been turned off the dialysing apparatuses were left undisturbed until the bouillon in them had cooled down to about 50° C. Then the dialysers were taken out, into each test-tube was poured a few c. c. of saliva previously cooled in the ice chest. The tubes were pushed down so that the membrane was immersed in the bouillon and after being left immersed for a very few minutes the tubes were taken out of the flasks and the latter closed with a pluck of aseptic cotton.

After the test-tubes had been withdrawn the flasks were put into the incubator and examined from day to day by macroscopical inspection. Those in which the bouillon becomes clouded are infected by bacteria, or mould. They are, therefore, not available for our experiments. Some, however, remain clear and if a few c. c. of their contents be withdrawn by a sterile pipette it will be found that the clear dialysate has a very strong diastatic action. To prove that the dialysate is absolutely non-contaminated it is necessary to pour a number of plates. If they remain sterile we know that the dialysate is free from bacteria, etc.; and in it we have a medium from which we can propagate the ptyalin granules from generation to generation.

From a dialysate prepared and tested for its non-contaminated character according to the methods indicated, a number of propagations were made. Inoculating with a platinum needle from the dialysate to a test-tube containing 10 c. c. of sterile glycerine-peptone-bouillon, and then after 24 hours to another test-tube No. 2, and so forth until No. 10 had been inoculated from No. 9. The tubes were kept in the incubator at 46° C. till 5 days after No. 10 had been inoculated. Then the bouillons were added to sterile starch-pastes. (40 c. c. paste containing 1 gram starch.) The pastes were then left in the incubator for about 8 days.

Result: Starch converted in all ten flasks. Titration of No. 10 gives 0.481 grams of maltose present. Iodine test—violet to brown; reaction slightly alkaline.

EXPERIMENT VIII

The next experimental series was undertaken to ascertain the optimum temperature of amylolytic action of the ptyalin granules. These and the following comparative experiments were made in the following manner:

Equal weights of starch were added to 100 ccm. of water in flasks and heated to boiling over a Bunsen burner. The contents of the flasks were continually shaken while the temperature rose in order to get a perfectly homogeneous starch solution. If the experiment was made to allow an action of the ferment lasting only for minutes a few seconds of boiling were considered sufficient; if a long continued action of the ferment was to be investigated, the flasks were sterilized for two hours. In ascertaining the optimum temperature one gram of cornstarch was added to 100 ccm. of distilled water. The starch solution so prepared was exposed to the action of one ccm. of saliva for ten minutes; after the lapse of this time the contents of the bottle were brought quickly to boiling over a Bunsen burner in order to arrest every further fermentation. The saliva used was all from a single crop diluted with nine times its volume of distilled sterile water. It was kept at the temperature of the room and not previously heated to the temperature of the experiment. The bottles upon the contents of which the ferment was to act were kept in a water bath and as soon as the desired temperature was reached the saliva was added and the temperature was kept constant for ten minutes. At the expiration of that time the contents of the bottle were quickly brought to boiling. The bottle was then set aside, the next one taken and treated like it, and when all had been so treated the amounts of sugar formed were ascertained by titration.

The following results were obtained:

Action of 1 ccm. saliva during 10 min. upon 100 ccm. 1% Starch Solution

Temperature	Amt. of Sugar Produced
20° C.	0.081 grams
25° C.	0.089 grams
30° C	0.091 grams
37½° C.	0.101 grams
40° C.	0.110 grams
42° C.	0.115 grams
44° C.	0.126 grams
46° C.	0.130 grams
48° C.	0.113 grams
50° C.	0.091 grams
60° C.	0.074 grams
70° C.	0.048 grams

According to the results of this experiment the optimum temperature of the amylolytic action of the ptyalin granules is 46° C.

This is the same result as that obtained by Kjeldahl (l. c.) in 1879. It seems that Kjeldahl's observation has not yet found its way into medical literature, because the physiological text-books give all optimum temperature of 35° to 40° C. The above amounts of sugar are comparatively small, as will be seen from other experiments. This is to be attributed to the fact that a pure cornstarch-distilled water solution, no salts or other additions being made, and a saliva, part of a large amount obtained by all kinds of reflex stimuli, were used.

For the following

Experiment IX

A nutrient solution was prepared containing ingredients as follows:

1000 ccm. water (lake water)
1 gram peptone
10 grams glycerine
5 grams chloride of sodium
0.1 gram phosphate of sodium.

The last three mentioned substances were chemically pure. It may be here stated that peptone, probably in consequence of impurities, has a weak copper reducing power itself. The peptone used was, therefore, tested in this respect. It was found that its reducing power was at least 100 times weaker than that of maltose, while the reducing power of the glycerine used was from two to three times weaker than that of the peptone used in this experiment.

The small sources of errors arising, from the use of these compounds, may be neglected, as the following figures will show. The following amounts of sugar were ascertained for

A. A 2% starch solution in water.
B. A 2% starch solution in the nutrient fluid.

Action of the ferment, 10 minutes.

Temperature	A.	B.
37½° C.	0.550 grams of sugar	1.158 grams of sugar
40° C.	0.773 grams of sugar	1.481 grams of sugar
46° C.	0.836 grams of sugar	1.603 grams of sugar
50° C.	0.700 grams of sugar	1.375 grams of sugar

The fact that the addition of peptones to a starch solution very much favors and hastens the action of the amylolytic ferment was first noticed by Chittenden and Smith (l. c.).

The saliva used in this experiment was from a small amount obtained without any artificial stimuli.

From the following

Experiments X and XI

It will be seen that a filtrate of saliva or malt diastase obtained by filtering through a Pasteur filter is very much weaker than the unfiltered saliva or malt diastase. This fact, also noted for the latter by Krabbe (l. c.), is alone sufficient to prove that the amylolytic ferments ptyalin and malt diastase are not bodies soluble in water, because if they were a Pasteur filtrate ought to be quite as strong, quite as active as a non-filtered preparation; because a body really held in a solution will pass the Pasteur filter without any diminution in concentration. I have prepared malt-extract; have ascertained its amount of sugar; have then filtered it through a Pasteur filter (taking care to pour off the first fluid collecting which goes over into the vacuum of the filter by condensation and not by filtration); and again ascertained the amount of sugar. There was, of course, no loss of the sugar held in solution, but the filtrate was very much reduced in amylolytic power. This is the case because the amylolytic ferments are not in solution; they are composed of cells, floating in the water; and evidently the major part of these cells is kept back by the filter and does not pass its pores; therefore, a filtrate is so very much weaker in amylolytic action. This could not be the case were the ferment truly unformed and truly soluble in water. It is, however, neither, but formed and insoluble.

From the saliva used in the foregoing experiments a few ccm. were filtered through a Pasteur filter and made to act upon 100 ccm. of a 2% starch solution in the nutrient fluid (B). The amounts of sugar obtained were:

Temperature	Amount of Sugar
37½° C.	0.226 grams
40° C.	0.257 grams
46° C.	0.280 grams
50° C.	0.179 grams

(The amount obtained at 50° C. was comparatively too small, because the amount of saliva left to be added was somewhat less than 1 ccm.)

With malt diastase the following figures were obtained:

5 minutes' action of 2 ccm. of diastase at 65° upon 100 ccm. of a 2% starch solution in water, 0.160 grams.

15 minutes upon 100 ccm. of a 2% starch solution in water, 0.206 grams.

5 minutes upon 100 ccm. of a 2% starch solution in water, (+ peptone), 0.220 grams.

15 minutes upon 100 ccm. of a 2% starch solution in water (+peptone), 0.296 grams.

Action of 2 ccm. from a Pasteur filtrate of which the stationary amount of sugar had been ascertained before and after filtration: 15 minutes' action upon a 2% starch solution (+ peptone) at 65° C., 0.062.

EXPERIMENT XII

It can be shown that one does not even have to filter through a Pasteur filter, but that filtration through a good dense paper filter suffices to weaken somewhat the diastatic power of the saliva. The experiments to demonstrate this were undertaken in connection with an arrangement to demonstrate that diluting saliva with sterile water increases the diastatic power; that is to say, if we take, on the one hand, one c. c. of saliva and add it to a starch paste diluting it (as is necessary to measure off an exact amount) right before we have it act upon the starch; and if we take on the other hand one c. c. of saliva, which has been diluted, a period of time before we add it to the starch; it will be found that the amylolytic power is greater in case No. 2 than in case No. 1.

The explanation for this observation is very simple. The saliva as we get it directly after secretion contains so many ferment granules that they cannot very much increase in number, but if we dilute the saliva the conditions for a more rapid multiplication are more favorable and the proteids and salts contained in the saliva furnished an excellent nutrient material.

The following experiments were made:

100 c. c. of saliva were collected under the usual precautionary measures. About half of this amount was kept in the ice chest as secreted, the other half was filtered in the ice chest through a paper filter made from a double layer of dense filter paper. When a filtrate had been obtained, the filtered, as well as the unfiltered, saliva was again divided off in equal measures. One-half of the filtered saliva and one-half of

the unfiltered secretion were diluted with nine times their volumes of sterile distilled water. Then all four flasks containing

A. unfiltered undiluted saliva
B. unfiltered diluted saliva
C. filtered undiluted saliva
D. filtered diluted saliva

were kept for two hours at the temperature of the room (30° C.). After the lapse of this time the two undiluted specimens were quickly diluted with nine times their volume of sterile distilled water and now ten c. c. of each of the four kinds of preparations were added to 90 c. c. of starch-paste (containing two grams of starch and 5 c. c. of a peptone-glycerine-bouillon).

The flasks were all kept in a water bath at 50° C. At the expiration of 12 minutes the water in the bath was quickly brought to boiling and kept boiling for some time. After cooling the contents of the flasks were examined for maltose by titration:

Results:

A. Unfiltered saliva, undiluted
    flask No. 1 amt. of sugar..........................0.802 grams
    flask No. 2 amt. of sugar..........................0.811 grams
B. Unfiltered saliva, diluted
    flask No. 1 amt. of sugar..........................0.975 grams
    flask No. 2 amt. of sugar..........................0.963 grams
C. Filtered saliva undiluted
    flask No. 1 amt. of sugar..........................0.588 grams
    flask No. 2 amt. of sugar..........................0.592 grams
D. Filtered saliva, diluted
    flask No. 1 amt. of sugar..........................0.637 grams
    flask No. 2 amt. of sugar..........................0.642 grams

The filter through which the saliva had been filtered was left in the ice chest for thirty-six hours and during that time sterile water was allowed to run through all the time. When it was finally taken out, perfectly dry, it was folded up and put into a flask containing 100 c. c. of a 2% starch paste. After three hours the starch became converted into dextrins and sugar. So evidently the filter, in spite of being washed for more than twenty-four hours, still had retained some of the alleged soluble ferment.

It can, of course, not be supposed that the filter can keep back and retain any of the small free ferment cells, but a number of these cells are found in the saliva not in a free state, but still in the interior of larger cells derived from the parenchyma of the salivary glands and some of these larger cells can be kept back by a double layer of dense filtering paper.

EXPERIMENT XIII

In view of the fact that the presence of peptone in a starch solution very materially increases the amount of sugar formed in a given time, the question arises: Does the diastatic ferment manufacture any sugar from peptone or does the presence of the latter only stimulate the action of the ferment? The possibility that the diastatic ferment granules can manufacture sugar from peptone had to be considered. Seegen[67] claims that in the animal body sugar is formed in the liver from peptones. I imagined that if the amylolytic ferment should have the power to manufacture sugar from peptone it would be done by the intermediary formation of starch or dextrins. As was noticed by Sachs many years ago, an observation confirmed by subsequent observers, there occurs in the plant a formation of starch from oils, and this starch is converted later on into sugar. We know also that yeast cells growing in an albuminous medium manufacture from albuminoid substances the yeast glycogen, a body belonging to the starch group. This glycogen is giving rise to the "self-fermentation" of the yeast cells in the absence of nutritive material and is first converted into sugar and this is subsequently split up into alcohol and CO_2.

All these observations suggest the possibility of peptones being, through several intermediate stages, converted into sugar. To test the ptyalin granules for such a possible converting power, peptone-bouillon was inoculated with saliva. The iodine test was made first every fifteen minutes, then every hour, finally from day to day. At no time could any starch or dextrins be discovered and the final product of this inoculation did not contain any substance fermentable with yeast cells.

EXPERIMENT XIV

The next experiments were undertaken with a view to ascertain the action of bichloride of mercury upon the development of the ptyalin cells.

1st Series

Action of 2 ccm. of saliva upon 100 ccm. of a 2% starch solution at 46° C. during 15 minutes.

Proportion of $HgCl_2$ in solution	Amount of sugar obtained
0	0.707 grams
1:50,000	0.524 grams
1:10,000	Traces (?)
1:5,000	0
1:2,000	0

2nd Series

Action of 2 ccm. saliva on 100 ccm. of a 2% starch solution (distilled water to exclude all other influences).

The solution used in this experiment was very carefully examined for any amount of sugar contained previous to the action of the ptyalin.

100 ccm. of this solution boiled with 2 ccm. of Fehling's solution did not noticeably reduce the copper hydrate. So 100 ccm. of the solution contained very much less than the 15.4 mgr. of sugar (maltose) which it would take to reduce the copper salt in 2 ccm. of Fehling's solution. The flasks used in this experiment were treated in the following manner, to get results as exact as possible: To No. 1 were added 2 ccm. of saliva, then No. 1 was transferred into the incubator (temp. 40° C.). After 3 minutes, 2 ccm. of saliva were added to No. 2, then No. 2 was put into the incubator, and so on. Exactly one hour after No. 1 had been put into the incubator it was taken out and its contents heated to boiling. After 3 minutes No. 2 was so treated and so on. The following were the results obtained:

Action of 2 ccm. saliva on 100 ccm. 2% starch sol. during one hour at 40° C.

No. Flask	Proportion of $HgCl_2$ in Solution	Amount of sugar obtained
1	0	1.305 grams
2	1:200,000	1.525 grams
3	1:100,000	1.603 grams
4	1: 80,000	1.674 grams
5	1: 20,000	0.128 grams

From these two series of experiments it appears that an addition up to 1:10,000 of corrosive sublimate to a starch solution absolutely prevents any amylolytic action and probably destroys the amylolytic granules in a short time. The addition of 1:20,000 is still very damaging because the yield in sugar is less than 1/10 what it is without the addition of sublimate; an addition of 1:50,000 still has a very marked retarding influence. Smaller additions, however, ranging from 1:80,000 to 1:200,000 favor and hasten the amylolytic action; in other words, quicken the multiplication of the cells. These ferment granules in this respect behave analogously to the yeast cells (Biernacki l. c.).

3rd Series

According to Chittenden and Cummins (l. c.) $HgCl_2$ even in very minute quantities, always influences unfavorably the action of the salivary ferment upon starch. So the above results do not agree with those obtained by those careful and trustworthy observers. Chittenden and Painter (l. c.) have, however, demonstrated that the presence of more or less proteid matter decidedly influences the effect of small additions of $HgCl_2$ to the fermenting solutions. They found that in 100 c. c. of a 1% starch solution which contained 10 c. c. of saliva and 0.001 or 1:100,000 $HgCl_2$, the latter had no hindering influence. In my above experiments the flasks used had been used before in bacteriological work and possibly in spite of a very thorough cleaning yet contained traces of proteid matter derived from bouillons formerly kept in these flasks.

The following experiment was made with peptone and $HgCl_2$ in the starch solution.

80 c. c. of water with varying percentages of $HgCl_2$.
10 c. c. of a peptone (2%) glycerine (2%) bouillon.
10 c. c. of a 20% saliva mixture (8 c. c. water, 2 c. c. saliva).

Action of the ferment in 40 minutes:

$HgCl_2$ in solution	Amount of sugar formed
0	1.167 grams
1:100,000	1.176 grams
1: 90,000	1.150 grams
1: 80,000	1.158 grams
1: 70,000	1.184 grams
1: 60,000	1.193 grams

The result bears out the statement of Chittenden and Painter (l. c.) that "the action of a given percentage of mercuric chloride can be considered as constant only for a given mixture under definite conditions." It seems to me very probable that under the conditions of the above series of experiments there was no $HgCl_2$ as such present in solution but a mercury albuminate.

EXPERIMENT XV

The following are the results of experiments made to ascertain the thermal death point of the ptyalin granules. Saliva exposed for one hour to a temperature of 72° to 73° C. lost every trace of amylolytic converting power.

Saliva left for 24 hours in the open air while the temperature was ranging from minus 5° C. to minus 15° C. and thawed three times during these 24 hours had likewise lost all converting power over starch.

Saliva exposed for one hour to a temperature of 70° to 71° (one must try to keep the temperature always at 70° and never go above 71° C.) changes starch-paste only to erythrodextrin, possibly to achroodextrin, but does not form any sugar.

On February 24, 1895, a test-tube containing about 10 c. c. of saliva was kept for forty minutes in a water bath at 70° C., this saliva was then added to a flask containing about 400 c. c. of a 4% starch-paste. The contents of the flask were examined on May 23; i. e., three months later. Starch liquefied, supernatant fluid clear opalescent; reaction neutral. The iodine test demonstrates the presence of a good deal of amylodextrin and erythrodextrin; 0.285 grams of maltose per 100 c. c. present.

On February 24, 1895, a test-tube containing about 10 c. c. saliva was kept for 40 minutes in a water bath at a temperature of 60°-61° C. Addition to starch paste like above; examination May 23; reaction neutral; small amount of amylodextrin, large amounts of erythrodextrin and achroodextrin, 0.806 grams maltose per 100 c. c.

On February 24 a test-tube containing about 10 c. c. of saliva was kept for 12 minutes in a water bath at a temperature of 74° to 75° C. The saliva was then added to a thick starch-paste (about 5%). Examination on June 15; reaction acid, the iodine test of a filtrate demonstrates a good deal of amylodextrin, small amounts of erythrodextrin and achroodextrin; maltose 0.192 grams per 100 c. c. These experiments show that saliva may be exposed to temperatures higher than is ordinarily supposed without destroying completely the amylolytic ferment. One must wait only long enough to demonstrate this fact and take care to exclude as much as possible the influence of microorganisms.

EXPERIMENT XVI

When the influence of the direct rays of the sun upon microorganisms was first studied, the impression seems to have prevailed that the influence of direct insolation was always inimical to the development of microorganisms. It has, however, been later shown that this is by no means universally the case; on the contrary we know now that some microorganisms are influenced favorably, others unfavorably by direct sunlight.

In order to study the influence of the direct rays of the sun upon amylolytic fermentation I first made very dilute starch solutions (½ gram starch to 100 c. c. water, adding 1 c. c. of saliva), and exposed some flasks to the rays of the sun while others were kept in the shade. A thermometer kept in a flask standing in the shade showed 26° C. while in a flask kept in the sun the temperature rose in 15 minutes to 29° C. In the flasks kept in the sunlight the amounts of sugar formed were found to be larger than the amounts ascertained for the flasks kept in the shade. The evidence afforded by this experiment could in no way be looked upon as conclusive in any sense. The difference in temperature was to be considered and more especially the fact that a ½% starch-paste is by no means a transparent medium, every part of which can be reached by the rays of the sun. It was, therefore, necessary to proceed in a different manner. To several hundred c. c. of a 5% starch-paste kept at a temperature of 65° C. about 100 c. c. of fresh saliva were added and the temperature was kept for ten minutes at 65° and then slowly raised to the boiling point. Under the conditions of this process of fermentation, it could be expected that the product would contain large quantities of dextrins and a comparatively small amount of sugar. After having been boiled for some minutes the starch-paste, which had become perfectly liquefied, was filtered and the amount of maltose ascertained to be 1.711 grams per 100 c. c. A number of flasks containing each 40 c. c. of the above filtrate were prepared and to each was added 10 c. c. containing ½ c. c. fresh saliva. Three flasks were kept in the shade, three were exposed to the rays of a hot summer sun, shining from a clear sky.

Result of the experiment: Time of fermentation, 10 minutes. Amount of saliva added ½ c. c.

Temperature in the flasks in the shade at the beginning of the experiment, 30° C.; at end of the experiment, 30° C.

Temperature in the flasks exposed to the sunlight, beginning of the experiment, 30° C.; end of experiment, 40° C.

Total amount of sugar ascertained for 100 c. c.:

Flask No. 1 (in shade), 3.080 grams	Flask No. 1 (exposed), 3.850 grams
Flask No. 2 (in shade), 3.080 grams	Flask No. 2 (exposed), 3.850 grams
Flask No. 3 (in shade), 3.080 grams	Flask No. 3 (exposed), 3.850 grams

This result certainly does not show any damaging influence of the direct rays of the sun. The larger amounts of sugar ascertained for the exposed flasks is probably not to be attributed to a favorable influence of the rays of light, but to the more favorable (higher) temperature.

The following arrangement likewise proves that the direct rays of the sun do not exert any destructive influence over the ptyalin granules, nor do they disturb the propagation of them.

Saliva was collected as usual and diluted with sterile, distilled water. The amount prepared was then equally divided into two flasks, one was kept in the shade, the other one exposed to the rays of a hot summer sun, shining from a clear sky. From time to time 10 c. c. of the dilute saliva were added to 100 c. c. of a 2% starch-paste. The saliva in the shade was kept at a temperature of 40° C., the temperature in the exposed saliva finally was up to 44° C.

The following amounts of sugar were formed from the starch-pastes: Time of fermentation, 10 minutes.

From the protected saliva Time having elapsed since saliva was collected		From the exposed saliva Time having elapsed since saliva was collected	
0 hours	1.000 grams	0 hours	1.013 grams
2 hours	1.055 grams	2 hours	1.242 grams
4 hours	1.262 grams	4 hours	1.400 grams
5 hours	1.375 grams	5 hours	1.581 grams

These series of experiments show that the rays of the sun have certainly no detrimental influence, possibly even a favorable influence.

The results also show very clearly again that saliva after having been diluted increases in amylolytic power.

In conclusion I want to refer to a constituent of mixed saliva not mentioned, as it seems, in most of the physiological text-books. Mixed saliva contains sugar. Kjeldahl (l. c.) ascertained for the saliva he used in his experiments an amount of sugar equal to 0.294%. I have repeatedly examined mixed saliva taken from my buccal cavity and have found amounts of sugar ranging from 0.2% to 0.401%. The latter high percentage was found in a saliva collected shortly after a meal, a saliva which was, before being examined, left in the incubator (40° C.) for thirty minutes, in order to convert every trace of starch (derived from victuals in which the starch has already been subjected to high temperatures and consequently can be quickly converted) into sugar.

In order to avoid any misunderstanding I want to state that I certainly do not want to be understood as meaning that saliva as secreted by the salivary glands contains sugar. All I want to state is that practically mixed saliva collected from the buccal cavity contains a small amount of sugar because we must expect small particles of food stuffs of the carbohydrate class to be present in the mouth more or less according to the kind of meal taken and to the length of time which has elapsed since this meal was taken.

PROPAGATION OF PTYALIN GRANULES DIRECTLY FROM THE SALIVARY GLANDS

A considerable number of experiments were made to propagate ptyalin granules directly from salivary glands. In order not to extend this paper too much I shall confine myself to describing briefly only the method employed to attain the object desired.

Salivary glands of cats and rabbits, preferably the parotids of rabbits, were extirpated under all aseptic precautions. The glands were at once, after being severed from their connections, dropped into sterile alcohol. This alcohol can, of course, not be prepared in a steam sterilizer, but it must be obtained by distillation into a sterile flask. In this medium the glands remained for several days; they were then taken out between the blades of sterile scissors, and dropped into sterile starch-pastes or bouillon. At the moment of dropping the glands into a culture medium it is well to cut them in two with the scissors. The alcohol so hardens the tissues that if the gland is not cut when being dropped into the culture media it takes a considerable time before a decided propagation of ptyalin granules with manifestation of amylolytic action is noticed.

One experiment may be here given:

Experiment XVII

Parotid of a rabbit extirpated under aseptic precautions, then left in sterile alcohol for 10 days, was then dropped into a glycerine-peptone-bouillon containing ½% carbolic acid (carbolic acid has a comparatively weak hindering influence upon the amylolytic ferment cells). After 5 days an inoculation was made to a test-tube, containing 10 c. c. of sterile bouillon. After 24 hours test-tube No. 2 was inoculated from No. 1 and so forth. Test-tube No. 8 was kept in the incubator for eight days. Its 10 c. c. bouillon was then added to 40 c. c. of starch-paste (containing 1 gram of starch).

Examination of the product of fermentation:

Iodine test: violet-brown; reaction: slightly alkaline; maltose: 0.653 grams; bacteria, etc.: no colonies developed on plates infected from tube No. 8.

CONCLUDING REMARKS

From the facts collected from the writings of various investigators and from my own experiments reported in the first part of this paper, it appears that our conception, heretofore universally held, as to the dead unorganized nature of the enzymes cannot be sustained, unless we admit that those enzymes are bodies not bound as to their mode of action by the fundamental laws of physics and chemistry; more especially not bound by the laws of the conservation and correlation of energy. In the second part of my paper I have undertaken to furnish the experimental evidence that the enzymes malt diastase and ptyalin are made up of exceedingly minute round corpuscles multiplying under suitable conditions, not only in the grain of malt and in the alveolar secretory salivary cells, but also outside of their natural abode under suitable conditions, and producing in the presence of starch as products of their metabolism, dextrins and maltose.

This experimental evidence was furnished by propagation experiments of such kind as we generally employed in bacteriological researches. In all these experiments the microscopic proof of the presence of the small ferment corpuscles, the size of which is very much inferior to that of any known microorganisms—was relegated to a secondary position, while the main evidence of the multiplication and propagation of these cells from generation to generation was furnished by the chemical proof of the presence of the well-known fermentation products of diastatic action upon starch; i. e., dextrins and maltose.

I have somewhere above expressed my belief in the identical nature of the diastase and ptyalin ferment corpuscles, a belief which in view of their identical chemical products under similar conditions seems to me to be fully justified. There are, however, some slight differences as to the action of the two enzymes diastase and ptyalin. The main difference is in the optimum temperature of their action, and there are probably also some slight differences as to favorable and inimical influences of some chemical agents increasing or hindering by their presence "ceteris paribus," the ferment action of the enzymes named, upon starch. These differences, I do not doubt, must be attributed to a variation in type between the ptyalin and diastase corpuscles. In view of the extreme minuteness of the ferment corpuscles, one is, of course, unable to state wherein this difference lies. Whatever may be the case, I think we have a right to assume that the ptyalin and the diastase corpuscles have a common progenitor, and that the differentiation manifesting itself in some slight distinguishing characters has been brought about in the course of time by the same factors that are responsible for the differentiation and development in the entire realm of organic life. If such a differentiation has been accomplished it is in itself a proof that those corpuscles, extremely minute and simple as they are, possess individual variability as well as any higher organism. It seems to me important to lay great stress upon this point.

That the cell could not be taken as the ultimate morphological and physiological unit of life is a view which has become more and more impressed upon the biologist for the last few decades. C. O. Whitman[68] with reference to this point recently stated: "We have long been aware that the cell could not be taken as the ultimate unit of life and every notable effort to account for heredity has led to the postulation of primary elements in comparison with which the cells appear as complex organisms. Since Ernst Brucke first contended for the organization of the cell in 1861 and the existence of smallest parts as the basis of this organization, we have seen similar ideas reappear in the "physiological units" of Herbert Spencer, the "gemmules" of Darwin; etc. We will find the secret of organization, growth, development, not in cell formation, but in those ultimate elements of living matter, for which "idiosomes" seems to me an appropriate name. What these idiosomes are, and how they determine organization, form, and differentiation is the problem of problems on which we must wait for more light. All growth, assimilation, reproduction and regeneration may be supposed to have their seat in these fundamental elements. They make up all living matter, are the bearers of heredity, and the real builders of the organism. Their action and control are not limited by cell boundaries."

Watase[69] in an article concerning the same subject expresses himself in the following manner:

"The behavior of the cytoplasm, thread or net-work suggests that it is formed of a group of small living particles, each with the power to assimilate, to grow and multiply by division. The chromosome in the same way is itself a colony of minute organisms of another kind, each endowed with similar attributes of vitality. The media in which they live—the cytolymph and cayolymph—are the media in which they breathe, from which they derive their nourishment, or within which they deposit the products of their metabolism. The reason why the cell as a whole assimilates, grows and divides is ultimately due to the fact that the minute particles which compose the cytoplasm and chromosome are endowed with these functions."

O. Hertwig[70] in giving a resume of his work on the cell says: "We are forced to the conclusion that the cell is a highly organized body, composed of numerous minute, different parts and that hence it is in itself to a certain extent a small elementary organism. It is, of course, necessary to consider that the hypothetical idioblasts must possess the power of multiplying by means of division like the higher elementary units, the cells . . . . Darwin, Naegeli and DeVries, therefore, logically assume that their gemmulæ, particles of idioplasm, and pangenæ are both able to grow and divide. This assumption enables us to draw another conclusion about the nature of the idioblasts, viz.: that by their very nature they cannot be identical with the atoms and molecules of the chemist and physicist, for the former are indivisible and the latter although divisible split up into portions, which no longer possess the properties of the whole. . . . . On this account idioblasts are not identical with the plastidules, the existence of which is assumed by Elsberg and Haeckel."

Weissner[71] says: "The assumption that protoplasm contains organized separate particles, which are capable of division, and that, in fact, it entirely consists of such living dividing particles is forced upon us as the result of recent inquiry. By means of the division of these particles growth is brought about and all the vital processes occurring in the organism depend upon them. They must, therefore, be considered the true elementary organs of life."

The above quotations demonstrate how keenly it has been felt that the cell could not be the ultimate morphological or physiological unit and that there must be something very much simpler than a cell, something possessing the powers of assimilation, growth, development, and multiplication.

I am convinced, as stated before, that the ptyalin and diastase ferment corpuscles are a true type of the elementary corpuscles looked for; and if it be true as Pasteur claimed years ago, that every vital process is one of fermentation, then it is to be assumed that each and every one of the different species of elementary corpuscles is a ferment cell like the diastase and ptyalin cells with very definite fermentation products as the consequence of its metabolism. Under this assumption and with our knowledge of the influence of the accumulation of the fermentation products upon the further development and propagation of ferment organisms and enzymes, many phenomena of cell life heretofore observed but not clearly understood would be brought nearer to our conception.

While the phenomena of heredity have forced such men as Darwin, Spencer, Naegeli, and others to assume the existence of minute corpuscles capable of multiplying in the cell, the demonstration of the existence of the character of such corpuscles as ferment corpuscles will bring many phenomena of heredity within the reach of a perfect understanding.

I have already stated my belief that the ptyalin and diastase ferment granules possess individual variability and I have made some few experiments, not reported in this paper, which seem to indicate that the ptyalin ferment granules may be adapted to new optimum temperatures just as bacteria can be adapted in this manner. If this can be demonstrated satisfactorily, then there cannot be any doubt that the ferment granules do possess individual variability which in the struggle for existence under altered conditions must bring about the survival of the fittest and the fixation of a variety more or less different from its ancestral progenitors. This being the case we can understand why the ferment granules of one being should be, and must, to a certain degree, be different from the ferment granules of another being of the same species, and as the germinal cells, respectively the ovum and the spermatozoa, derive their ferment granules from their parental organism or organisms, the fact that the offspring inherits the parental morphological and physiological properties even to the extent of characters of individual variability can be traced back to the difference in character of these ferment granules, acquired under varied conditions and fixed by the general laws of organic development.

The question may be asked right here whether the ovum and the spermatozoa do possess all the ferment granules of the parental organism. If the ovum is to mature outside of the maternal organism such must be the case, lest the conditions of the existence of the embryo permit of the acquisition of certain ferment cells, necessary to its full development by symbiotic additions from the outside. If, however, the embryo matures in the maternal body there is no absolute necessity why the ovum should possess all ferment cells necessary to the organism postpartum. The embryo developing in the maternal organism may through the placental circulation be supplied with a number of species of ferment granules necessary to it in its after life, but not present in the ovum.

We know that certain infectious diseases, such as small-pox, in the human race, may be transmitted intra utero from the mother to the offspring. And as Virchow has shown that all pathological processes have their physiological analoga, it is reasonable to assume that the intrauterine infection from mother to embryo, by a pathological ferment corpuscle, through the placental circulation, shows us what also occurs physiologically with reference to the ferment granules necessary to build up the complete mature embryo. As far as the ptyalin corpuscles are concerned there would be no difficulty in assuming this, since we know that a number of fluids of the animal body, among which is the blood of higher races, possess slight diastatic properties doubtless depending upon the presence of ptyalin granules.

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